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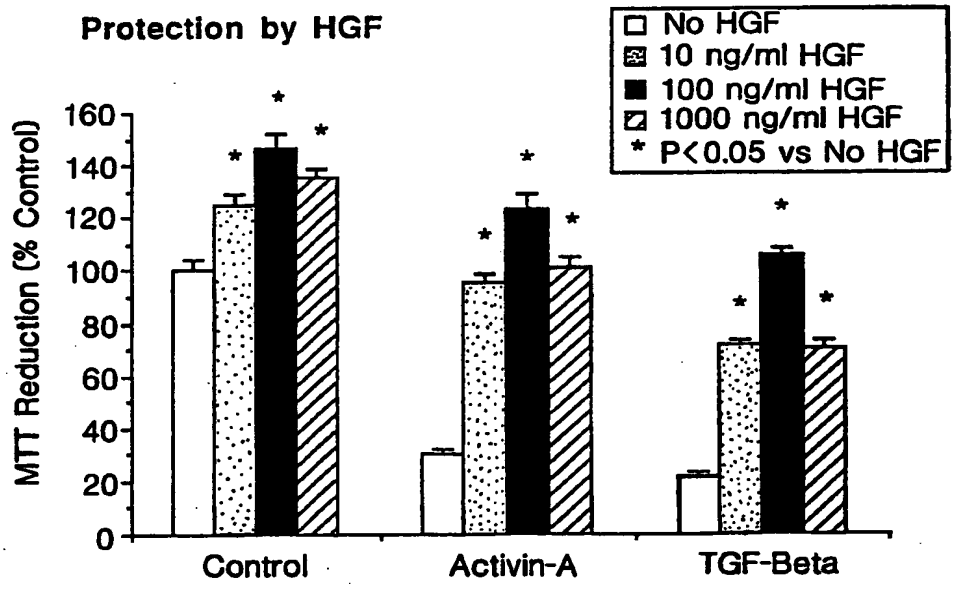

(57) Abstract

The invention concerns the use of HGF in the prevention of the establishment or of the progress of liver damage in patients at risk of developing or having been diagnosed with liver damage.

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Protection Against Liver Damage by HGF

Field of the Invention

The present invention concerns the use of hepatocyte growth factor (HGF) for the prevention of liver damage.

Background Art

Liver damage occurs in a number of acute and chronic clinical conditions, including drug-induced hepatotoxicity, viral infections, vascular injury, autoimmune disease and blunt trauma. In addition, patients subject to inborn errors of metabolism may be at risk for developing liver damage. Symptoms of liver damage occurring as a result of these clinical conditions include, for example, fulminant hepatic failure with cholestasis, hepatic lesions, and liver tissue necrosis, and in many instances, the restoration of normal liver function is vital to the survival of patients.

Hepatotoxic compounds can induce almost all types of liver injury (Benhamou, J-Pierre, Liver Cells and Drugs, Chapter 164, pgs. 3-12, Colloque INSERM/John Libbey Eurotext Ltd., edited by A. Guillozo (1988)). The susceptibility of the liver to damage by chemical agents may be related to its primary role in drug metabolism or is a consequence of hypersensitivity reactions. Up to 25% of cases of fulminant hepatic failure may be the result of adverse reactions to medical agents. Hepatotoxic compounds are also an important cause of chronic liver disease including fatty liver, hepatitis, cirrhosis and vascular and neoplastic lesions of the liver. (Sinclair *et al.*, Textbook of Internal Medicine, 569-575 (1992) (editor, Kelley; Publisher, J.B. Lippincott Co.).

Hepatotoxic compounds may induce liver damage by cytotoxicity to the liver directly or through the production of toxic metabolites (this category includes the hypersensitivity reaction which mimics a drug allergy); cholestasis, an arrest in the flow of bile due to obstruction of the bile ducts; and vascular lesions, such as in veno occlusive disease (VOD), where injury to the vascular endothelium results in hepatic vein thrombosis. Individual susceptibility to liver damage induced by hepatotoxic compounds is influenced by genetic factors, age, sex, nutritional status, exposure to other drugs, and systemic diseases (Sinclair *et al.*, Textbook of Internal Medicine, Supra). Hepatotoxic compounds known to induce liver damage include acetaminophen, nitrosoureas, used in the treatment of cancer, and isoniazid, used in the treatment of tuberculosis.

Although in minor liver damage induced by hepatotoxic compounds, withdrawal of the causative agent may be sufficient to substantially reverse the damage occurred, in many instances where fulminant hepatic failure ensues, aggressive medical therapy, including the administration of antidotes, such as N-acetylcysteine, may be required. A antidotal treatment is, however, often not effective when given more than about 10-24 hours after exposure to the hepatotoxic compound (Goodman and Gilman's, The Pharmacological Basis of Therapeutics 8th edition, Gilman *et al.*, Pergamon Press, pp. 658-659 (1990)). If this

happens, the liver damage may become permanent and life thr atening, leaving liver transplantation as the only remedy.

Radiation therapy can also induce liver damage. It has been shown that hypoalbuminemia and decreased hepatic blood flow, b th symptoms of liver damage, occur after single-dose total body irradiation (Moulder, J. *et al.* Int J Radiat Oncol Biol Phys 19: 1389-1396 (1990)). Awwad, H. *et al.*, Int J Radiat Oncol Biol Phys 19(5): 1229-1232 (1990) show that lung and hepatic toxicities constitute the main radiation-related damage after half-body irradiation used as the treatment for patients with non-Hodgkin's lymphomas and recommend low dose-rate or multifraction irradiation in order to reduce the risk of liver toxicity. McCracken, J. *et al.*, Cancer Treat Rep 69(1): 129-31 (1985) caution that combined radiotherapy and intra-arterial chemotherapy may result in significant chronic liver damage, as monitored by serum enzyme levels, and recommend exercising caution in the future use of the therapy. Fajardo, L. *et al.* Arch Pathol Lab Med 104(11): 584-8 (1980) show that radiation-induced liver disease is characterized structurally by progressive fibrous obliteration of central veins (VOD) and that in several patients, VOD occurred at radiation doses conventionally considered safe.

Inborn errors of metabolism exist which result in liver damage. Patients who have a genetically limited capacity to convert aryl epoxides to nontoxic dihydriols, seem predisposed to developing liver damage from exposure to phenytoin and halotane, drugs useful as anesthetics. Also, susceptibility to contraceptive steroid-associated cholestasis appears to have a strong genetic component (Sinclair *et al.*, Textbook of Internal Medicine, Supra).

Liver damage of any origin can be diagnosed and monitored by biochemical tests of liver markers, such as assessment of hepatic blood flow or prothrombin clotting time, or serum markers, such as serum bilirubin, serum transaminase, and serum alkaline phosphatase levels and (Cornelius, C., Hepatotoxicology pg. 181, (1991) and (Awwad, H. Int J Radiat Oncol Biol Phys 19(5): 1229-1232 1990)). Liver damage can also be monitored from histological evaluation of liver tissue, which is helpful in determining the type and extent of liver damage (Sinclair, S. Textbook of Internal Medicine, Supra. It is known that results from *in vitro* biochemical tests measuring liver function or serum markers and/or results from liver tissue biopsy, correlate with *in vivo* liver damage assessment. Often, a combination of biochemical tests, tissue biopsy, patient medical history, and assessment of means inducing liver damage is used in determining the extent of liver damage.

Liver cell (hepatocyte) regeneration is believed to be controlled by various growth stimulatory and growth inhibitory cytokines of autocrine or paracrine origin, however, the exact role and action mechanism of these factors is far from entirely understood.

In vitro, DNA synthesis in isolated hepatocytes has been shown to be stimulated by growth factors such as epidermal growth factor (EGF) and type a transforming growth factor (TGF-α) and to be inhibited by interleukin 1β (IL-1β) (Nakamura et al., Exp. Cell Res., 179:

488-497 (1988)), transforming growth factor β1 (TGF-β1) (Braun *et al.*, Proc. Natl. Acad. Sci. USA, 85: 1539-1543 (1988); Nakamura *et al.*, Biochem. Biophys. Res. Comm., 133: 1042-1050 (1985); Carr *et al.*, Cancer Res., 46: 2330-2334 (1986); Castilla *et al.*, New Eng. J. Med., 324: 933-940 (1992); Houck *et al.*, J. Cell. Physiol., 135: 551-555 (1988); Strain *et al.*, Biochem. Biophys. Res. Commun., 145: 436-442 (1987)), and activin (PCT Publication No. WO92/22321). TGF-β1 has been shown to inhibit *in vivo* DNA synthesis taking place after partial hepatectomy. Russell *et al.*, Proc. Natl. Acad. Sci. USA, 85: 5126-5130 (1988). Vascular endothelial growth factor (VEGF), an endothelial cell mitogen, is expressed in the normal liver (Berse, *et al.*, Mol. Biol. Cell, 3(2): 211-220 (1992)), where it plays a role in tissue nutrition and waste removal.

More recently, a further protein, named hepatocyte growth factor (HGF) has been shown to be a complete mitogen for primary hepatocytes. Although based upon the observation that the level of HGF in the serum rapidly increases following experimental damage to the liver and in patients with fulminate hepatic failure it has been proposed that HGF may be an important mediator of liver regeneration *in vivo*, and certain experimental evidence supports this hypothesis, there is no clear consensus among scientists about the role of HGH in liver regeneration. Rosen *et al.*, Cell Growth and Differentiation 2: 603 (1991) caution that markedly elevated HGF levels in patients with chronic liver disease may indicate that HGF is a marker for or instigator of human liver damage rather than a repair factor.

Growth factors, proteins with growth factor-like activities, such as cytokines, (Andus *et al.*, Hepatology 13(2): 364-375 (1991)) and therapeutics, such as tissue plasminogen activator (Baglin, *et al.*, Bone Marrow Transplant 5(6): 439-441 (1990)), have been indicated in the treatment of liver damage.

HGF was purified by Nakamura *et al.* from the serum of partially hepatectomized rats (Biochem. Biophys. Res. Comm. 122: 1450-1459 (1984)). Subsequently, HGF was purified from rat platelets, and its subunit structure was determined (Nakamura *et al.*, Proc. Natl. Acad. Sci. USA, 83, 6489-6493 (1986); and Nakamura *et al.*, FEBS Letters 224, 311-316 (1987)). The purification of human HGF (hHGF) from human plasma was first described by Gohda *et al.*, J. Clin. Invest. 81, 414-419 (1988). According to the results reported by Gohda *et al.* hHGF is more effective in the stimulation of cultured hepatocyte proliferation than human epidermal growth factor (hEGF) or insulin, and the effect of hHGF with the maximal effects of hEGF and insulin is "additive or synergistic". Similarly, Zarnegar *et al.*, Cancer Research 49, 3314-3320 (1989) described the purification of a polypeptide growth factor, called human hepatopoietin A (HPTA) having very similar properties to hHGF as characterized in earlier publications. As the authors do not disclose the amino acid sequences of their purified proteins, the degree of the structural similarity between the two factors can not be determined.

The N-terminal amino acid sequence of rabbit HPTA was described by Zarnegar *et al.*, Biochem. Biophys. Res. Comm. 163, 1370-1376 (1989).

Both rat HGF and hHGF have been molecularly cloned, including the cloning and sequencing of a naturally occurring variant lacking 5 amino acids in the Kringle 1 (K1) domain, designated "delta5 HGF" (Miyazawa *et al.*, Biochem. Biophys. Res. Comm. 163: 967-973 (1989); Nakamura *et al.*, Nature 342: 440-443 (1989); Seki *et al.*, Biochem. and Biophys. Res. Commun. 172: 321-327 (1990); Tashiro *et al.*, Proc. Natl. Acad. Sci. USA 87: 3200-3204 (1990); Okajima *et al.*, Eur. J. Biochem. 193: 375-381 (1990)). The sequences reported by Miyazawa *et al.* and Nakamura *et al.* for hGH differ at several positions. The comparison of the amino acid sequence of rat HGF with that of hHGF revealed that the two sequences are highly conserved and have the same characteristic structural features. The length of the four Kringle domains in rat HGF is exactly the same as in huHGF. Furthermore, the cysteine residues are located in exactly the same positions; an indication of similar three-dimensional structures (Okajima *et al.*, Supra; Tashiro *et al.*, Supra).

A naturally occurring hHGF variant has recently been identified which corresponds to an alternative spliced form of the hHGF transcript containing the coding sequences for the N-terminal finger and first two kringle domains of mature hHGF (Chan *et al.*, Science 254: 1382-1385 (1991); Miyazawa *et al.*, Eur. J. Biochem. 197: 15-22 (1991)). This variant, designated HGF/NK2, has been proposed to be a competitive antagonist of mature hHGF.

The HGF receptor has been identified as the product of the c-Met proto-oncogene (Bottaro *et al.*, Science 251: 802-804 (1991); Naldini *et al.*, Oncogene 6: 501-504 (1991)), an 190-kDa heterodimeric (a disulfide-linked 50-kDa a-chain and a 145-kDa β-chain) membrane-spanning tyrosine kinase protein (Park *et al.*, Proc. Natl. Acad. Sci. USA 84: 6379-6383 (1987)). The c-Met protein becomes phosphorylated on tyrosine residues of the 145-kDa β-subunit upon HGF binding.

The levels of HGF increase in the plasma of patients with hepatic failure (Gohda *et al.*, Supra) and in the plasma (Lindroos *et al.*, Hepatol. 13: 734-750 (1991)) or serum (Asami *et al.*, J. Biochem. 109: 8-13 (1991)) of animals with experimentally induced liver damage. The kinetics of this response is rapid, and precedes the first round of DNA synthesis during liver regeneration suggesting that HGF may play a key role in initiating this process. Although HGH was originally thought to be a liver-specific mitogen, more recently, it has been shown to be a mitogen for a variety of cell types including melanocytes, renal tubular cells, keratinocytes, certain endothelial cells and cells of epithelial origin (Matsumoto *et al.*, Biochem. Biophys. Res. Commun. 176: 45-51 (1991); Igawa *et al.*, Biochem. Biophys. Res. Commun. 174, 831-838 (1991); Han *et al.*, Biochem. 30: 9768-9780 (1991); Rubin *et al.*, Proc. Natl. Acad. Sci. USA 88: 415-419 (1991)). Interestingly, HGF can also act as a "scatter factor", an activity that promotes the disassociation of epithelial and vascular endothelial cells in vitro (Stoker *et al.*, Nature 327: 239-242 (1987); Weidner *et al.*, J. Cell

Biol. 111: 2097-2108 (1990); Naldini *et al.*, EMBO J. 10: 2867-2878 (1991)). Moreover, HGF has recently been described as an epithelial morphogen (Montesano *et al.*, Cell 67: 901-908 (1991)). Therefor , HGF has been postulated to be important in tumor invasion and in embryonic development. Chronic c-Met/HGF receptor activation has been observed in certain malignancies (Cooper *et al.*, EMBO J. 5: 2623 (1986); Giordano *et al.*, Nature 339: 155 (1989)).

Activin consists of a homodimer or heterodimer of inhibin β subunits, which may be β_A or β_B subunits. Vale *et al.*, Recent Prog. Horm. Res., 44: 1-34 (1988). There is 95-100% amino acid conservation of β subunits among human, porcine, bovine, and rat activins. The β_A and β_B subunits within a given species are about 64-70% homologous.

The activin β_A and β_B homodimers ("Activin A" and "Activin B," respectively) have been identified in follicular fluid, and both molecules have been cloned and their genes expressed. Mason *et al.*, Biochem. Biophys. Res. Commun., 135: 957 (1986); EP Pub. No. 222,491 published May 20, 1987; Mason *et al.*, Molecular Endocrinol., 3: 1352-1358 (1989); Schwall *et al.*, Mol. Endocrinol., 2: 1237-1242 (1988); Nakamura et al., J. Biol. Chem., 267: 16385-16389 (1992). The complete sequence of the β_B subunit is published in Serono Symposium Publications, entitled "Inhibin- Non-Steroidal Regulation of Follicle Stimulating Hormone Secretion", eds. H.G. Burger *et al.*, abstract by A.J. Mason et al., vol. 42, pp. 77-88 (Raven Press, 1987), entitled "Human Inhibin and Activin: Structure and Recombinant Expression in Mammalian Cells." The recombinant molecule has been shown to increase serum levels of FSH in rats when delivered by subcutaneous injection. Schwall *et al.*, Endocrinol., 125: 1420-1423 (1989); Rivier and Vale, Endocrinol., 129: 2463-2465 (1991).

Activin was initially identified in follicular fluid as a naturally occurring gonadal peptide involved in the regulation of the secretion of follicle-stimulating hormone (FSH) by rat anterior pituitary cells. Vale *et al.*, Nature, 321: 776-779 (1986); Ling *et al.*, Nature, 321: 779-782 (1986); DePaolo *et al.*, Proc. Soc. Exp. Biol. Med., 198: 500-512 (1991); Ying, Endocrine Rev., 9: 267-293 (1988).

Subsequent studies of activin revealed other activities, including the effects on follicular granulosa cell differentiation (Sugino *et al.*, Biochem. Biophys. Res. Commun., 153: 281-288 [1988]), spermatogonial proliferation (Mather *et al.*, Endocrinol., 127: 3206-3214 [1990]), erythroid differentiation (EP Publ. No. 210,461 published February 4, 1987; Eto *et al.*, Biochem. Biophys. Res. Commun., 142: 1095-1103 [1987]; Murata *et al.*, Proc. Natl. Acad. Sci. USA, 85: 2434-2438 [1988]; Yu *et al.*, Nature, 330: 765-767 [1987], stimulation of insulin secretion by pancreatic islets (Totsuka *et al.*, Biochem. Biophys. Res. Commun., 156: 335-339 [1988]), enhancement of proliferation of fibroblast (Hedger *et al.*, Mol. Cell Endocrinol., 61: 133-138 [1989]), stimulation of glucose production by hepatocytes (Mine *et al.*, Endocrinology, 125: 586-591 [1989]), induction of a dose-dependent increase in inositol phosphates in rat parenchymal liver cells, an effect also seen with EGF (Mine et al.,

Biochem. Biophys. Res. Comm., 186: 205-210 [1992]), modulation of somatotroph functions (Billestrup *et al.*, Mol. Endocrin. I., 4: 356-362 [1990]), modulati n of nerve cell diff rentiation (Schubert *et al.*, Nature, 344: 868-870 [1990]; Hashimoto *et al.*, Biochem. Biophys. Res. Comm., 173: 193-200 [1990]), and mesoderm induction. Smith *et al.*, Nature, 345: 729-731 (1990); Mitrani *et al.*, Cell, 63: 495-501 (1990).

It has also been found that chronic renal failure serum contains as much activin as normal serum, but the difference between normal serum and the serum of patients with renal failure exists in the context of a specific inhibitor of activin, with the suggestion that activin could be utilized in the therapy of the anemia of such patients. Shiozaki *et al.*, Biochem. Biophys. Res. Commun., 183: 273-279 (1992). While these activities have been demonstrated *in vitro*, the role of activin *in vivo* remains poorly understood.

Inhibin and activin are members of a family of growth and differentiation factors. The prototype of this family is TGF-β (Derynck et al., Nature, 316: 701-705 (1985)), which, according to one source, also possesses FSH-releasing activity (Ying et al., Biochem. Biophys. Res. Commun., 135: 950-956 (1986). Other members of the TGF-β family include the Mullerian inhibitory substance, the fly decapentaplegic gene complex, and the product of Xenopus Vg-1 mRNA.

TGF-β1 appears to be a negative regulator of liver growth, and the TGF-β molecule is associated with regression of other epithelial tissues in the embryo (Silberstein and Daniel, Science, 237: 291-293 [1987]) or adult (Kyprianou and Isaacs, *supra*) and of certain cancers. Kyprianou *et al.*, Cancer Res., 51: 162-166 (1991). Recently, it was reported that cell proliferation and apoptosis are coordinately regulated by TGF-β1 in cultured uterine epithelial cells. Rotello *et al.*, Proc. Natl. Acad. Sci. USA, 88: 3412-3415 (1991). Apoptosis is a physiological cell death wherein the nucleus condenses and the cytoplasm fragments.

Studies *in vivo* showed that apoptotic hepatocytes in normal and prenoeplastic liver exhibited immunostaining for TGF-β1. Oberhammer *et al.*, Naunyn-Schmiedeberg's Arch. Pharmacol. Suppl., 343: R24 (1991). See also Oberhammer *et al.*, Cancer Res., 51: 2478-2485 (1991). Evidence has now been found that hepatocyte death induced by TGF-β1 *in vitro* is indeed apoptosis. Oberhammer *et al.*, Proc. Natl. Acad. Sci. USA, 89: 5408-5412 (1992).

A new class of gonadal protein factors, named follistatin or FSH-suppressing protein (FSP), has been isolated from side fractions derived from purifying porcine and bovine ovarian inhibins and activins. Ying, Endoc. Rev., 9: 267-293 (1988); Ling *et al.*, "Isolation and characterization of gonadal polypeptides that regulate the secretion of follicle stimulating hormone," in Hodgen *et al.*, eds., Non-Steroidal Gonadal Factors: Physiological Roles and Possibilities in Contraceptive Development, Jones Institute Press, Virginia, (1988), pp. 30-46. Follistatin was initially characterized by its ability to suppress FSH secretion from the pituitary. The action of follistatin is apparently similar to that of inhibin, but structurally the two

proteins are quite different. Ueno *et al.*, Proc. Natl. Acad. Sci. USA, 84: 8282-8286 (1987); Robertson *et al.*, Biochem. Biophys. Res. Commun., 149: 744-749 (1987).

Follistatin is a glycosylated single-chain protein that is found in forms having molecular weights ranging from 31 to 39 kDa. All of these forms have similar amino acid compositions and identical amino-terminal amino acid sequences. The molecular cloning of cDNA with the gene of follistatin revealed two forms, a smaller molecular weight form and a larger form, which are generated by alternative splicing. The smaller form represents a carboxy-terminal truncated form of the larger precursor.

Recent examinations of follistatin gene expression in rat tissues have shown that follistatin mRNA is detected not only in the gonads but also in the kidney, decidual tissue, pancreas, cerebral cortex, pituitary, *etc*. Shimasaki *et al.*, Mol. Endocrinol., 3: 651-659 (1989); Kaiser *et al.*, Endocrinology, 126: 2768-2770 (1990); Michel *et al.*, Biochem. Biophys. Res. Comm., 173: 401-407 (1990).

It has been found that follistatin is able to neutralize the diverse actions of activin in various systems such as stimulation of FSH secretion by cultured pituitary cells (Kogawa *et al.*, Endocrinology, 128: 1434-1440 [1991]) and induction of mesodermal tissue formation in *Xenopus* oocytes. Asashima *et al.*, Arch. Dev. Biol., 200: 4-7 (1991). It has been found, in fact, that immunoreactive follistatin is widespread in rat tissues, including hepatic cells, which demonstrated homogeneous immunoreactivity from moderate to strong. Kogawa *et al.*, Endocrinol. Japan, 38: 383-391 (1991). The authors suggest that follistatin is a ubiquitous protein regulating a wide variety of activin actions.

There exists a need for an effective method for the prevention of liver damage. This need exists in any patient population in which chronic or acute liver damage has been or can potentially be induced, for example by hepatotoxic compounds, radiation exposure, viral infection, autoimmune disease, elevated *in vivo* levels of proteins, including liver cell growth inhibitory proteins, hepatotoxic proteins and cytokines, or genetic factors, and where it is desirable to inhibit the progression of such damage. This need further exists in a patient population at risk of developing liver damage, such as in the case of drug overdose, in the case of accidental exposure to infected blood samples, or in a clinical scenario which includes aggressive chemotherapy or radiation therapy. In many instances, the treatment of serious, life threatening conditions, such as cancer, is severely limited by the hepatotoxicity of the chemotherapeutic agents and/or radiation therapy employed. It would be desirable to be able to expose patients to higher doses of such chemotherapeutics or radiation therapy for an extended period of time without the risk of severe liver damage. There is a related need for an effective liver damage preventative agent which could be included in a clinical protocol potentially inducing liver damage.

It would be particularly desirable to provide means for the prevention of the further progression of liver damage in situations where early intervention is critical. This would be

particularly beneficial when known antidotes are no longer effective because of the time elapsed since the exposure to the causative factor of liver damage.

Accordingly, it is an object of the present invention to provide means for the prevention of liver damage in patients at risk of developing liver damage, especially due to hepatotoxic compounds, radiation, or genetic predisposition. It is another object to provide means for the prevention of the progression of liver damage already occurred.

It is a further object to enable the extended exposure of patients to potentially hepatotoxic treatments and/or to increase the dose of such treatments by preventing the (further) development of liver damage.

It is a still further object of the present invention to provide means for early intervention in patients showing symptoms of at risk of developing liver damage.

It is another object to provide means for preventing the progression of liver damage at a time when antidotes known in the art would no longer be effective.

These and further objects will be apparent to one of ordinary skill in the art.

Summary of the Invention

The present invention is based on the experimental finding that HGF provides effective protection from anticipated liver damage due to the administration of a hepatotoxic compound, and in particular, from anticipated liver tissue necrosis and anticipated elevated serum enzyme levels, both indicative of liver damage. The present invention is also based on the experimental finding that HGF provides protection from activin and TGF-β induced cell death in hepatocytes. We have further found that HGF is capable of preventing the progression of liver damage already occurred. Although HGF has been associated with hepatocyte regeneration, its ability to prevent the occurrence or further progression of liver damage is entirely unexpected.

In one aspect, the present invention relates to a method for the prevention of the establishment of liver damage in a patient at risk of developing liver damage comprising administering to the patient a preventatively effective amount of hepatocyte growth factor (HGF). The patient preferably is mammalian, more preferably human. Potential or actual liver damage may be due to numerous external or internal factors, including intentional or accidental exposure to a hepatotoxic compound, radiation exposure, genetic predisposition, autoimmune disease and viral infections of the liver.

The invention further concerns a method for the prevention of the progression of liver damage already occurred.

In another aspect, the invention concerns a composition comprising a therapeutically effective amount of a hepatotoxic therapeutic agent and a liver damage preventative amount of HGF.

In a further embodiment, the invention relates to a method for the treatment of a patient with a hepatotoxic therapeutic agent effective in the prevention or treatment of a disorder or pathologic physiological condition, comprising:

a) administering to said patient, simultaneously or in optional order, a biologically effective dose of said therapeutic agent and a preventatively effective amount of HGF,

b) monitoring said patient for indication of liver damage, and

c) continuing said treatment until said disorder or condition is eliminated or until liver damage is indicated.

In yet a further embodiment, the present invention relates to a method for the prevention of the establishment or progress of liver damage in a patient at risk for developing or having been diagnosed with viral or autoimmune hepatitis comprising administering to said patient a liver damage preventative amount of HGF.

Brief Description of the Drawings

Figure 1 shows the alkaline phosphatase (ALP), alanine transaminase (ALT), aspartate aminotransferase (AST), γ-glutamine transpeptidase (GGT) enzyme levels, total bilirubin and amylase in rats treated with BiCNU® and recombinant human HGF (rHGF) as compared with those treated with BiCNU® or vehicle alone. The treatments were performed as described in Example 1.

Figure 2 (A) shows hepatocellular necrosis with accompanying hemorrhage which extends from the portal triad almost to the central vein in a rat treated with BiCNU®. Figure 2 (B) shows the lack of hepatocellular necrosis in the liver of an rhHGF-treated, BiCNU® exposed rat. The treatments were performed as described in Example 1.

Figure 3 shows HGF protection against liver damage induced by Activin-A or TGF-β as measured by MTT reduction by the method of Carmichael et al. (Cancer Res 47:936-942 [1987]).

Detailed Description of the Invention

The phrase "liver damage" is used herein in the broadest sense, and indicates any structural or functional liver injury resulting, directly or indirectly, from internal or external factors or their combinations. Liver damage can be induced by a number of factors including, but not limited to, exposure to hepatotoxic compounds, radiation exposure, mechanical liver injuries, genetic predisposition, viral infections, autoimmune disease, such as, autoimmune chronic hepatitis and as a result of elevated *in vivo* levels of proteins, such as activin and TGF-β.

Liver damage induced by hepatotoxic compounds includes direct cytotoxicity including drug hypersensitivity reactions, cholestasis, and injury to the vascular endothelium (Sinclair *et al.*, Textbook of Internal Medicine, Supra).

A number of hepatotoxic compounds, including certain therapeutics, induce cytotoxicity. Hepatotoxic compounds can produce liver cytotoxicity by direct chemical attack

or by the production of a toxic metabolite. Although the exact mechanism of hepatotoxicity is uncertain, the products of reductive metabolism are highly reactive species that bind to cellular macromolecules and cause lipid peroxidation and inactivation of drug metabolizing and other enzymes. The membrane injury provokes release of calcium from mitochondria and smooth endoplasmic reticulum and appears to interfere with the calcium ion pump, which normally prevents cytosolic accumulation of calcium. The deleterious effect on cell metabolism with resultant calcium accumulation, the loss of potassium and enzymes from the cytoplasm, and the loss of essential energy that results from mitochondrial injury all contribute to the necrosis of hepatic tissue.

Many hepatotoxic compounds unpredictably produce liver damage in a small proportion of recipients. In some patients, the liver damage is referred to as a hypersensitivity reaction and is like that of a drug reaction, where the patient presents with fever, rash and eosinophilia and has a recurrence of symptoms upon rechallenge of the drug. In other situations, the mechanism for injury is unknown and may represent aberrant metabolism in susceptible patients that permits the production or accumulation of hepatotoxic metabolites.

Those drugs inducing cytotoxicity by direct chemical attack include the following:
Anesthetics, such as, Enflurane, Fluroxene, Halothane, and Methoxyflurane;
Neuropsychotropics, such as, Cocaine, Hydrazides, Methylphenidate, and Tricyclics;
Anticonvulsants, such as, Phenytoin and Valproic acid;
Analgesics, such as, Acetaminophen, Chlorzoxazone, Dantrolene, Diclofenac, Ibuprofen, Indomethacin, Salicylates, Tolmetin, and Zoxazolamine;
Hormones, such as, Acetohexamide, Carbutamide, Glipizide, Metahexamide, Propylthiouracil, Tamoxifen, Diethylstilbestrol;
Antimicrobials, such as, Amphotericin B, Clindamycin, Ketoconazole, Mebendazole, Metronidazole, Oxacillin, Paraaminosalicylic acid, Penicillin, Rifampicin, Sulfonamides, Tetracycline, and Zidovudine;
Cardiovascular drugs, such as, Amiodarone, Dilitiazem, a-Methyldopa, Mexiletine, Hydrazaline, Nicotinic acid, Papaverine, Perhexiline, Procainamide, Quinidine, and Tocainamide; and
Immunosuppressives and Antineoplastics, such as, Asparaginase, Cisplatin, Cyclophosphamide, Dacarbazine, Doxorubicin, Fluorouracil, Methotrexate, Mithramycin, 6-MP, Nitrosoureas, Tamoxifen, Thioguanine, and Vincristine; and
Miscellaneous drugs, such as, Disulfiram, Iodide ion, Oxyphenisatin, Vitamin A and Paraaminobenzoic acid.

Those hepatotoxic compounds producing hypersensitivity reaction in the liver include the following:
Phenytoin, Paraamino salicylic acid, Chlorpromazine, Sulfonamides, Erythromycin estolate, Isoniazid, Halothane, Methyldopa, and Valproic acid.

Hepatotoxic compounds inducing cholestasis, an arrest in the flow of bile, may take several forms. Centribular cholestasis is accompanied by portal inflammatory changes. Bile duct changes have been reported with some drugs such as erythromycin, while pure canalicular cholestasis is characteristic of other drugs such as the anabolic steroids. Chronic cholestasis has been linked to such drugs as methyltestosterone and estradiol.

Those hepatotoxic compounds inducing cholestatic disease include the following: Contraceptive steroids, androgenic steroids, anabolic steroids, Acetylsalicylic acid, Azathioprine, Benzodiazepine, Chenodeoxycholic acid, Chlordiazepoxide, Erythromycin estolate, Fluphenazine, Furosemide, Griseofulvin, Haloperidol, Imipramine, 6-Mercaptopurine, Methimazole, Methotrexate, Methyldopa, Methylenediamine, Methyltestosterone, Naproxen, Nitrofurantoin, Penicillamine, Perphenazine, Prochlorperazine, Promazine, Thiobendazole, Thioridazine, Tolbutamide, Trimethoprim-sulfamethoxazole, Arsenic, Copper, and Paraquat.

Some drugs, although primarily cholestatic, can also produce hepatoxicity, and therefore the liver injury they cause is mixed. The drugs causing mixed liver injury include, for example, the following:
Chlorpromazine, Phenylbutazone, Halothane, Chlordiazepoxide, Diazepam, Allopurinol, Phenobarbital, Naproxen, Propylthiouracil, Chloramphenicol, Trimethoprim-sulfamethoxazxole, Amrinone, Disopyramide, Azathioprine, Cimetidine, and Ranitidine.

Vascular lesions of the liver, including thrombosis of the hepatic veins, occlusion of the hepatic venules or veno occlusive disease (VOD), and peliosis hepatitis, can be produced by drugs. In addition, lesions including sinusoidal dilatation, perisinusoidal fibrosis, and hepatoportal sclerosis can occur. Midzonal and pericentral sinusoidal dilatation was first reported as a complication of oral contraceptive therapy. Peliosis hepatitis is a condition consisting of large blood-filled cavities that results from leakage of red blood cells through the endothelial barrier, followed by perisinusoidal fibrosis. It has been described in patients taking oral contraceptives, anabolic steroids, azathioprine and danazol. Injury and occlusion of the central hepatic venules is also known to be related to the ingestion of pyrrolizidine alkaloids, such as bush teas. The initial lesion is central necrosis accompanied by a progressive decrease in venule caliber. All of these lesions may be only partially reversible when the drug is stopped and cirrhosis can develop.

Several types of benign and malignant hepatic neoplasm can result from the administration of hepatotoxic compounds. Adenomas, a lesion restricted to women in the childbearing years, is related to the use of contraceptive steroids and the risk increases with duration of use. Hepatocellular carcinoma may also be seen in patients taking androgenic hormones for aplastic anemia or hypopituitarism.

Hepatotoxic compounds known to cause hepatic lesions include the following: Contraceptive steroids, Pyrriolizidine alkaloids, Urethane, Azathioprine, 6-Mercaptopurine, 6-Thioguanine, Mitomycin, BCNU, Vincristine, Adriamycin, Intravenous Vitamin E, Anabolic-

androgenic ster ids, Azathioprine, Medroxypr gesterone acetate, Estrone sulfate, Tamoxifen, inorganic arsenicals, Thorium dioxide, Vitamin A, methotrexate, Methylamphetamine hydrochloride, Vitamin A, Corticosteroids, Thorium dioxide, and Radium therapy.

Liver damage caused by other factors usually takes similar forms.

Liver damage, whether caused by the hepatotoxicity of a compound, radiation therapy, genetic predisposition, mechanical injury or any combination of such and other factors, can be detected by several means. Biochemical tests have been used clinically for many years as the standard measure of hepatotoxicity. Most biochemical tests generally fall into two categories: tests which measure specific liver markers, for example, prothrombin clotting time, and/or hepatic blood flow, or tests which analyze serum markers, for detection of necrosis, cholestasis, progressive fibrogenesis, or hepatoma (Cornelius, C. in Hepatotoxicology, Meeks *et al.* eds., pp. 181-185 (1991)). The importance of such tests lies in their simplicity and the fact that they are non-invasive. The rationale for the use of serum enzymes in assessing liver damage is that these enzymes, normally contained in the liver cells, gain entry into the general circulation when liver cells are injured. Elevated serum enzyme activity suggests necrosis and/or cholestasis. Elevated levels of serum bilirubin conjugates suggest intra or extra hepatic cholestasis. However, there are certain limitations for the use of serum enzyme levels as single means of diagnosing liver injury. Serum enzyme levels may increase as a result of leakage from cells with altered permeability due to systemic effects of an agent rather than specific liver injury caused by a chemical. Histopathological examination of the liver is the next logical step in identifying and quantitating the nature and extent of liver injury.

The serum enzymes as markers of liver injury can be divided into four groups based on specificity and sensitivity to liver damage (Kodavanti, *et al.* in Hepatotoxicology, Supra, pgs. 241-244).

Group I: these enzymes indicate more selectively hepatic cholestasis when elevated, e.g. alkaline phosphatase (AP), 5'-nucleotidase (5'-ND), and a-glutamyl transpeptidase (G-GT) and leucine aminopeptidase (LAP).

Group II: These enzymes indicate parenchymal injury when elevated, e.g., aspartate transaminase (AST), alanine transaminase (ALT), fructose-1,6-diphosphate aldolase (ALD), lactate dehydrogenase (LDH), isocitrate dehydrogenase (ICDH), ornithine-carbamoyl-transferase (OCT), and sorbitol dehydrogenase (SDH) arginase and guanase.

Group III: These enzymes represent injury of other tissue when elevated e.g., creatine phosphokinase (CPK).

Group IV: These enzymes are depressed in hepatic injury, e.g., cholinesterase (ChE).

Other serum markers include, procollagen type III peptide levels (PIIIP) to assess if hepatic fibrogenesis is active; ammonia blood levels in hepatoencephalopathies; ligand in levels in necrosis and hepatoma; hyaluronate levels due to hepatic endothelial cell damage;

a-1-fetoprotein (AFP) levels to detect hepatoma; carcinoembryonic antigen (CEA) levels to detect cancer metastasis to the liver; elevations of antibodies against a variety of cellular components, such as, mitochondrial, and nuclear and specific liver membrane protein; and detection f proteins, such as, albumin, globin, amino acids, cholesterol, and other lipids. Also, biochemical analysis of a variety of minerals, metabolites, and enzymes obtained from liver biopsies can be useful in studying specific biochemical defects in inherited, acquired, and experimentally induced liver disorders.

Liver function tests can be performed to assess liver injury. Liver function tests include the following:

Group I assessment of hepatic clearance of organic anions, such as, bilirubin, indocyanine green (ICG), sulfobromophthalein (BSP) and bile acids;

Group II assessment of hepatic blood flow by measurements of galactose and ICG clearance; and

Group III assessment of hepatic microsomal function, through the use of the aminopyrine breath test and caffeine clearance test.

For example, serum bilirubin can be measured to confirm the presence and severity of jaundice and to determine the extent of hyperbilirubinemia, as seen in parenchymal liver disease. Aminotransferase (transaminase) elevations reflect the severity of active hepatocellular damage, while alkaline phosphatase elevations are found with cholestasis and hepatic infiltrates (Isselbacher, K. and Podolsky, D. in Hartison's Principles of Internal Medicine, 12th edition, Wilson *et al.* eds., 2: 1301-1308 (1991)).

Methods for performing serum enzyme analysis are known in the art and are, for example, described in Kodavanti, *et al.*, Supra.

Because extensive liver injury may lead to decreased blood levels of albumin, prothrombin, fibrinogen, and other proteins synthesized exclusively by hepatocytes, these protein levels may be measured as indicators of liver injury. In contrast to measurements of serum enzymes, serum protein levels reflect liver synthetic function rather than just cell injury (Podolsky, D, Principles of Internal Medicine, 12th edition, Wilson *et al.* eds., 2: 1308-1311 (1991)).

In many patients, computed tomography (CT), ultrasound, scintiscans, or liver biopsy may be needed to determine the nature of the liver disease (Isselbacher, K, Supra and Friedman, L, and Needleman, L. in Harrison's Principles of Internal Medicine, 12th edition, Wilson *et al.* eds., 2: 1303-1307 (1991)).

The term "prevention" as used in the context of the present invention includes the complete or partial blocking of the occurrence of anticipated liver damage and the interception or moderation of the progress of liver damage already occurred. Whereas it is foreseen that existing liver damage may be completely or partially reversed, this is not a requirement under this definition.

The term "preventatively effective amount" is used to designate an amount effective in achieving prevention as hereinabove defined.

Patients "at risk of developing liver damage" include those patients who are anticipated to be exposed to or who have been exposed to any factor known to have the potential of inducing liver damage. This includes exposure to hepatotoxic compounds (whether as part of a therapy or due to accidental exposure), in doses conventionally considered safe or in doses conventionally considered unsafe, radiation, or any clinical therapy useful in the treatment of a disease, wherein said clinical therapy is known to induce liver damage. The definition further includes actual or potential sustained liver injury through physical trauma including, blunt trauma, gunshot wounds, or surgery. Patients at risk of developing liver damage include those patients having inborn errors of metabolism and who are genetically predisposed to induction of liver damage, or those mammalian patients susceptible to liver damage due to other risk factors including genetic factors, age, sex, nutritional status, exposure to other drugs, and systemic diseases. Patients at risk of developing liver damage also includes those patients who are anticipated to be exposed to or who have been exposed to viruses such as hepatitis A, B, C, D, or E, or autoimmune chronic hepatitis.

"Radiation" as used herein refers to exposure to x-rays or any other rays known to have hepatotoxic side-effects, including radiation therapy and accidental exposure.

In the context of the present invention the term "hepatocyte growth factor" or "HGF" is used to refer to a native hepatocyte growth factor or any fragment or derivative thereof capable of the prevention of the establishment or of the progress of liver damage as determined in standard tests as hereinabove described. The term specifically includes human and non-human, such as rat HGF, in mature, pre, pre-pro, or pro forms, purified from natural source, chemically synthesized or recombinantly produced, and their derivatives.

The term "human hepatocyte growth factor" or "hHGF" refers to a polypeptide encoded by the cDNA sequence published by Miyazawa, *et al.*, Supra, or Nakamura *et al.*, Nature, Supra, including its single- and double-chain, mature, pre, pre-pro, and pro forms, purified from natural source, chemically synthesized or recombinantly produced, or any fragment or derivative thereof, retaining the qualitative ability to prevent the establishment or of the progress of liver damage as determined by any of the standard tests described above.

The "native" "wild-type" hHGF cDNA encodes a 728 amino acids polypeptide (pre-pro hHGF) having a molecular mass (M_r) of about 82,000, and a heterodimeric structure, composed of a large α-subunit of 440 amino acids (M_r 69,000) and a small β-subunit of 234 amino acids (M_r 34,000). The nucleotide sequence of the hHGF cDNA reveals that both the α- and the β-chains are contained in a single open reading frame coding for a pre-pro precursor protein. In the predicted primary structure of mature hHGF, an interchain S-S bridge is formed between Cys 487 of the α-chain and Cys 604 in the β-chain (see Nakamura *et al.*, Nature,

Supra). The N-terminus of the α-chain is prec ded by 54 amino acids, starting with a m thionine group. This segment includes a signal sequence and the prosequence. The α-chain starts at amino acid (aa) 55, and contains four Kringle domains. The Kringle 1 domain extends from about aa 128 to about aa 206, the Kringle 2 domain is between about aa 211 and about aa 288, the Kringle 3 domain is defined as extending from about aa 303 to about aa 383, and the Kringle 4 domain extends from about aa 391 to about aa 464 of the a-chain. It will be understood that the definition of the various Kringle domains is based on their homology with kringle-like domains of other proteins (prothrombin, plasminogen), therefore, the above limits are only approximate. The HGF β-chain includes a serine-protease like domain. hHGF contains four putative glycosylation sites, which are located at positions 294 and 402 of the a-chain and at positions 566 and 653 of the β-chain. The sequences reported for native hHGF by Miyazawa *et al.* and Nakamura *et al.* differ in 14 amino acids. The reason for the differences is not entirely clear; polymorphism or cloning artifacts are among the possibilities. Both sequences are specifically encompassed by the term "native hHGF" as defined for the purpose of the present invention. The term specifically includes "delta5 hHGF", a variant in which 5 amino acids are deleted in the first kringle domain of native human hHGF, which was first identified and described by Seki *et al.*, Supra.

The term "derivative" is used to define amino acid sequence and glycosylation variants, and covalent modifications of a native hepatocyte growth factor.

Single-chain variants of HGF are described in PCT Application No. PCT/US93/04648 filed 17 May 1993. The single-chain variants are resistant to proteolytic cleavage by enzymes that are capable of *in vivo* conversion of the single-chain HGF proenzyme into its two-chain form. Absent alterations, the proteolytic cleavage takes place between Arg494 and Val495 of the wild-type hHGF sequence. The single-chain hHGF variants preferably have an alteration at or adjacent to amino acid positions 493, 494, 495 or 496 of thw wild-type hHGF amino acid sequence. In a preferred group, a smaller, apolar or acidic amino acid is substituted for arginine at position 494 to yield single-chain hHGF variants. Typical representatives of single-chain hHGF variants are, for example, R494A, R494D and R494E hHGF.

Protease domain variants of HGF are described in PCT Application No. PCT/US93/04648 filed 17 May 1993. Desirable HGF amino acid variants are those that have retained or enhanced receptor binding affinity as compared to the corresponding wild-type HGF. Variants which, in addition, exhibit substantially retained or increased biological activity as compared to the corresponding wild-type HGF (HGF agonists) are particularly preferred. The protease domain variants may, for example, comprise an alteration in a region corresponding to the catalytic site of serine proteases. In hHGF variants, the alteration preferably is at or adjacent to any of positions 534, 673 and 692 of wild-type hHGF. Typical protease-domain variants of hHGF include, but are not limited to, Q534H; Y673S; V692S; Q534H,Y673S; Y673S,V692S; Q534H,Y673S,V692S hHGF.

The foregoing patent applications also describe C-terminal truncation and kringle domain deletion variants of HGF. Such deletions may be combined with each other and/or with alterations within the protease domain or at or around the proteolytic cleavage site of a native HGF molecule.

Covalent derivatives of HGF include, but are not limited to, posttranslational modifications and derivatives obtained by reaction with organic derivatizing agents.

Other derivatives comprise those that are covalently bonded to a nonproteinaceous polymer. The nonproteinaceous polymer ordinarily is a hydrophilic synthetic polymer, i.e. a polymer not otherwise found in nature. However, polymers which exist in nature and are produced by recombinant or *in vitro* methods are useful, as are polymers which are isolated from nature. Hydrophilic polyvinyl polymers fall within the scope of this invention, e.g. polyvinylalcohol and polyvinylpyrrolidone. Particularly useful are polyvinylalkylene ethers such a polyethylene glycol, polypropylene glycol.

The HGF may be linked to various nonproteinaceous polymers, such as polyethylene glycol, polypropylene glycol or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

The HGF may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, in colloidal drug delivery systems (e.g. liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, 16th Edition, Osol, A., Ed. (1980).

An HGF sequence can be linked to a immunoglobulin constant domain sequence. The resultant molecules are commonly referred to as HGF immunoglobulin chimeras or immunoadhesins. Such chimeras can be constructed essentially as described in WO 91/08298 (published 13 June 1991).

For purposes herein, immunoadhesins are antibody-like molecules which combine the binding specificity of a protein such as a cell-surface receptor, a cell-adhesion molecule or a ligand (an "adhesin"), with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesins comprise a fusion of an amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site (antigen combining site) of an antibody (i.e. is "heterologous"), and an immunoglobulin constant domain sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous amino acid sequence comprising at least the binding domain of a receptor (including cell adhesion molecules) or a ligand.

Immunoadhesins can possess many of the valuable chemical and biological properties of human antibodies. Since immunoadhesins can be constructed from a human protein sequence with a desired specificity linked to an appropriate human immunoglobulin hinge and constant domain (Fc) sequence, the binding specificity of interest can be achieved using

entirely human components. Such immunoadhesins are minimally immunogenic to the patient, and are safe for chronic or repeated use.

In the HGF immunoglobulin chimera, ordinarily, the HGF sequence is fused C-terminally to the N-terminus of the constant region of an immunoglobulin in place of the variable region(s); however N-terminal fusions of the HGF sequence is also desirable. The immunoglobulin constant domain sequence in the HGF immunoglobulin chimeras or immunoadhesins may be obtained from any immunoglobulin, such as IgG-1, IgG-2, IgG-3, or IgG-4 subtypes, IgA, IgE, IgD or IgM.

Typically, such fusions retain at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain. This ordinarily is accomplished by constructing the appropriate DNA sequence and expressing it in recombinant cell culture. Alternatively, however, the HGF immunoglobulin chimeras or the immunoadhesin may be synthesized according to known methods.

The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion or binding characteristics of HGF or the immunoadhesin.

In a preferred embodiment, the C-terminus of a sequence which contains the binding site(s) for an HGF receptor, is fused to the N-terminus of the C-terminal portion of an antibody (in particular the Fc domain), containing the effector functions of an immunoglobulin, e.g. immunoglobulin G_1. It is possible to fuse the entire heavy chain constant region to the sequence containing the receptor binding site(s). However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site (which defines IgG Fc chemically; residue 216, taking the first residue of heavy chain constant region to be 114 (Kobet *et al.*, Supra), or analogous sites of other immunoglobulins) is used in the fusion. In a particularly preferred embodiment, the amino acid sequence containing the receptor binding site(s) is fused to the hinge region and CH2 and CH3 or CH1, hinge, CH2 and CH3 domains of an IgG_1, IgG_2 or IgG_3 heavy chain. The precise site at which the fusion is made is not critical, and the optimal site can be determined by routine experimentation.

Immunoadhesins reported in the literature include fusions of the T cell receptor[*] [Gascoigne *et al.*, Proc. Natl.Acad. Sci. USA 84, 2936-2940 (1987)]; CD4[*] [Capon *et al.*, Nature 337, 525-531 (1989); Traunecker *et al.*, Nature 339, 68-70 (1989); Zettmeissl *et al.*, DNA Cell Biol. USA 9, 347-353 (1990); Byrn *et al.*, Nature 344, 667-670 (1990)]; L-selectin (homing receptor) [Watson *et al.*, J. Cell. Biol. 110, 2221-2229 (1990); Watson *et al.*, Nature 349, 164-167 (1991)]; CD44[*] [Aruffo *et al.*, Cell 61, 1303-1313 (1990)]; CD28[*] and B7[*] [Linsley *et al.*, J. Exp. Med. 173, 721-730 (1991)]; CTLA-4[*] [Lisley *et al.*, J. Exp. Med. 174, 561-569 (1991)]; CD22[*] [Stamenkovic *et al.*, Cell 66. 1133-1144 (1991)]; TNF receptor

[Ashkenazi *et al.*, Proc. Natl. Acad. Sci. USA 88, 10535-10539 (1991); Lesslauer *et al.*, Eur. J. Immunol. 27, 2883-2886 (1991); Peppel *et al.*, J. Exp. Med. 174, 1483-1489 (1991)]; NP receptors [Bennett *et al.*, J. Biol. Chem. 266, 23060-23067 (1991)]; and IgE receptor *α*-chain* [Ridgway and Gorman, J. Cell. Biol. 115, abstr. 1448 (1991)], where the asterisk (*) indicates that the receptor is member of the immunoglobulin superfamily.

It is believed that any HGF molecule exhibiting HGF biological activity is suitable for the purpose of the present invention. Accordingly, the testing of HGF biological activity is indicative of the utility of an HGF derivative as a liver damage preventative agent.

The terms "biological activity", "biologically active", "activity" and "active" refer to any mitogenic, motogenic or morphogenic activities exhibited by wild-type human HGF. The HGF biological activity may, for example, be determined in an *in vitro* or *in vivo* assay of hepatocyte growth promotion. Adult rat hepatocytes in primary culture have been extensively used to search for factors that regulate hepatocyte proliferation. Accordingly, the mitogenic effect of an HGF variant can be conveniently determined in an assay suitable for testing the ability of an HGF molecule to induce DNA synthesis of rat hepatocytes in primary cultures. Adult rat hepatocytes in primary culture have been extensively used to search for factors that regulate hepatocyte proliferation, accordingly, techniques for isolating and culturing rat hepatocytes are well known in the art. Human hepatocytes can, for example, be obtained from whole liver perfusion on organs deemed unacceptable for transplantation, pare-downs of adult livers used for transplantation in children, fetal livers and liver remnants removed at surgery for other indications. Human hepatocytes can be cultured similarly to the methods established for preparing primary cultures of normal rat hepatocytes.

Hepatocyte DNA synthesis can, for example, be assayed by measuring incorporation of [^{3}H]thymidine into DNA, with appropriate hydroxyurea controls for replicative synthesis. Nuclear labelling is confirmed by autoradiography. A method for measuring hepatocyte DNA synthesis in primary culture of hepatocytes with or without aphidicolin is described by Nakamura *et al.*, in Biochem. Biophys. Res. Comm. 122(3): 140-1459 (1984), and in J. Biochem. 94: 1029-1035 (1983).

The effect of HGF on hepatocyte growth can also be tested *in vivo* in animal models of liver dysfunction and regeneration, such as in rats following partial hepatectomy, or carbon tetrachloride caused hepatic injury, in D-galactosamine induced acute liver failure models, etc. According to a suitable protocol, a liver poison, e.g. *α*-naphthylisothiocyanate (ANIT) is administered to rats in a predetermined concentration capable of causing reproducible significant elevation of liver enzyme and bilirubin levels. The rats are then treated with the HGF to be tested, sacrificed and the liver enzyme and bilirubin levels are determined. The livers are additionally observed for hepatic lesions.

The ability of HGF to liver damage can be best tested *in vivo* in transgenic animal models, such as described in U.S. 5,087,571 issued March 22, 1988. According to a

suitable protocol, transgenic animals subject to liver disease or liver damage are treated with the HGF to be tested or HGF co-administered with a therapeutic useful in the treatment of disease, sacrificed and the liver enzyme and bilirubin levels determined. The livers are additi nally observed f r hepatic lesions.

For purposes herein, "activin antagonist" refers to any molecule that inhibits the activity of activin in causing death of hepatocytes. As used herein, "activin" refers to homo- or heterodimers of β chains of inhibin, prepro forms, and pro forms, together with glycosylation variants thereof, whether in native form or synthetic or recombinant form. Activin A refers to activin with the two chains of β_A. Activin AB refers to activin with the chains β_A and β_B. Activin B refers to activin with the two chains of β_B.

Typically the activin antagonist is a protein that binds to an active site of activin and includes, *e.g.*, follistatin as described in Esch *et al.*, Mol. Endocrinol., 1: 849-855 [(1987); Shimasaki *et al.*, Proc. Natl. Acad. Sci. USA, 85: 4218-4222 (1988); Shimasaki *et al.*, Biochem. Biophys. Res. Comm., '152: 717-723 (1988); Shimasaki *et al.*, Mol. Endocrinol., 3: 651-659 (1989); Ueno *et al.*, Proc. Natl. Acad. Sci. USA, 84: 8282 (1987); Nakamura *et al.*, Science, 247: 836 (1990); Shimonaka *et al.*, Endocrinology, 128: 3313 (1991).

In addition, the antagonist may be a non-proteinaceous small molecule that acts as an activin antagonist. Such molecules can be screened by their ability to inhibit the action of activin in promoting liver injury or liver cell death using the assays described above and in the examples, such as the MTT assay.

The definition of antagonist also includes an anti-activin antibody, whether polyclonal or monoclonal. Monoclonal antibodies specific for human recombinant activin A or B can be produced as described by Corrigan *et al.*, Endocrinology, 128: 1682 (1991). Briefly, inbred HPG-hypogonadal mice (Jackson Laboratories, Wilmington, MA) are hyperimmunized in the hind footpad with purified recombinant activin A, B, or AB. Cells harvested from the draining lymph nodes are then fused with the mouse myeloma line X63-Ag8.653. Kearney *et al.*, J. Immunol., 123: 1548 (1979). The fusions are screened for reactivity and specificity in an ELISA using recombinant human activin A, activin B, activin AB, and inhibin A as coat proteins. Wong *et al.*, Clinical Chemistry, 36: 192 (1990). Parental hybridomas that react specifically with either recombinant human activin A, B, or AB are cloned by limiting dilution. Ascites fluids are produced in Balb/c nu/nu mice, and antibody is purified by protein A-sepharose affinity chromatography (Repligen Corp., Cambridge, MA) according to established procedures (Goding, J. Immunol. Meth., 20: 241 [1978]; Ey *et al.*, Immunochemistry, 15: 429 [1978]), and stored under sterile conditions in phosphate buffered saline (PBS) at 4°C. Antibodies against activin or activin peptides that may also be suitable herein, although they may also cross-react with inhibin to some degree, include those described by Lofgren *et al.*, J. Immunoassay, 12: 565 (1991); Shintani *et al.*, J. Immunol. Meth., 137: 267 (1991); Groome and Lawrence, Hybridoma, 10: 309 (1991); Groome, J. Immunol. Meth., 145: 65-69

(1992); and Schwall *et al.*, Non-Radi metric Assays: Technology and Application in Polypeptide and Steroid Hormone Detection, pages 205-220 (Alan R. Liss, Inc., 1988).

Another suitable activin antagonist herein is an inhibitor of activin such as that described in Shiozaki *et al.*, *supra*, or a soluble form of an activin receptor.

Examples of suitable activin receptors include that described in U.S. Patent No. 5,216126. Briefly, the receptor is described as not binding to TGF-β, having a molecular weight on reduced 10% SDS-PAGE of 135-150 Kd, and having an N-terminal sequence of: ValLeuThrGluGluThrGlulleIleMetProThrProLysProGluLeuXaaAlaXaaXaaAsn, wherein Xaa indicates an unknown amino acid. To the extent that the "activin receptor" described in Mathews and Vale, Cell, 65: 1-20 [1991] and Mathews *et al.*, Science, 255: 1702-1705 (1992) blocks activin biological activity in hepatocytes, it is included herein. Activin receptors have also been reported by Attisano *et al.*, Cell, 68: 97-108 [1992] and Kondo *et al.*, Biochem. Biophys. Res. Comm., 181: 684-690 [1991].

The definition of activin antagonists also includes fragments of the above molecules that contain the active site needed to block activin activity, including F(ab) and Fc fragments of antibodies, *etc.*

Efficacy in preventing cell death in certain liver diseases is seen with a treatment regimen that employs an activin antagonist administered in an effective dose.

Examples of TGF-β antagonists include antibodies to TGF-β such as those described in Lucas *et al.*, J. Immunol., 145: 1415-1422 (1990); Dasch *et al.*, J. Immunol., 142: 1536-1541 (1989); Ellingsworth *et al.*, J. Biol. Chem., 261: 12362-12367 (1986); Cheifetz *et al.*, Cell, 48: 409-415 (1987); Florini et al., J. Biol. Chem., 261, 16509-16513 (1986); Roberts et al., Proc. Natl. Acad. Sci. USA, 83: 4167-4171 (1986); Assoian and Sporn, J. Cell Biol., 102: 12178-1223 (1986); Ellingsworth et al., Cell. Immunol., 114: 41 (1988); Flanders et al., Biochemistry, 27: 739 (1988); Keski-Oja et al., Cancer Res., 47: 6451 (1988); Danielpour and Sporn, J. Cell Biochem., 13B: 84 (1989); and Danielpour et al., J. Cell Physiol., 138: 79-86 (1989).

Additional TGF-β antagonists that are suitable include non-proteinaceous small molecules that act as a TGF-β antagonist in blocking the ability of TGF-β to cause hepatic injury or hepatocyte death, screened by, e.g., the MTT test, and a soluble form of the TGF-β receptor or TGF-β binding protein of any type, as described, for example, in Lin et al., Cell, 68: 775-785 (1992); Lin *et al.*, J. Cell Biochem. Suppl., 16 Part B, p. 125 (1992); Wang *et al.*, Cell, 67: 797-805 (1991); EP 369,861 published 23 May 1990; Wang *et al.*, J. Cell Biochem. Suppl., 16, part B, p. 129 (1992); Lopez-Casillas *et al.*, Cell, 67: 785-795 (1991); O'Grady *et al.*, J. Biol. Chem., 266: 8583-8589 (1991); Segarini *et al.*, J. Biol. Chem., 267: 1048-1053 (1992); MacKay *et al.*, J. Biol. Chem., 265: 9351-9356 (1990); Cheifetz and Massague, J. Biol. Chem., 266: 20767-20772 (1991); Cheifetz and Massague, J. Cell Biochem. Suppl., 16, part B, p. 121 (1992); Ichijo *et al.*, J. Biol. Chem., 266: 22459-22464

(1991); Borisuth *et al.*, Invest. Ophthal. and Vis. Sci., 33: 596-603 (1992); Mitchell and O'Conn r-McCourt, J. Cell Biol., 115: 3, Part 2, p. 265A (1991).

For recent reviews of TGF-β receptors, see Segarini, "TGF-βReceptors," Clinical Applications of TGF-β (Wiley, Chichester [Ciba Foundation Symposium 157], p. 29-50, 1991), and Massague *et al.*, Annals NY Acad. Sci., p. 59-72, 1990. If antibodies to activin or TGF-β are employed as the antagonist, they are prepared by any suitable technique. For example, activin or immunogenic fragments of activin may be used to induce the formation of anti-activin antibodies, which are identified by routine screening. Similarly, TGF-β or immunogenic fragments of TGF-β may be used to induce the formation of anti-TGF-β antibodies which are identified by routine screening. Such antibodies may either be polyclonal or monoclonal antibodies, or antigen-binding fragments of such antibodies (such as, for example, F(ab) or $F(ab)_2$ fragments). The antibodies are monovalent or polyvalent for activin. An activin antagonist or mixtures thereof or with another suitable adjuvant therapeutic agent is generally used in a single course of therapy.

Polyclonal antibodies to activin or TGF-β generally are raised in animals by multiple subcutaneous (s.c.) or intraperitoneal (i.p.) injections of the activin polypeptide together with an adjuvant. It may be useful to conjugate the activin antigen polypeptide (including its chains and fragments containing the target amino acid sequence) to a protein that is immunogenic in the species to be immunized, *e.g.*, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N = C = NR$, where R and R^1 are different alkyl groups.

The route and schedule for antibody stimulation of the host animal or cultured antibody-producing cells therefrom are generally in keeping with established and conventional techniques for antibody stimulation and production. While mice are frequently employed as the test model, it is contemplated that any mammalian subject including human subjects or antibody-producing cells obtained therefrom can be manipulated according to the processes of this invention to serve as the basis for production of mammalian, including human, hybrid cell lines.

Animals are typically immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1 μg of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5 to 1/10 the original amount of conjugate in Freund's incomplete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. Seven to 14 days later animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same activin polypeptide, but conjugated to a different protein and/or

through a different cross-linking agent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response. Monoclonal antibodies are prepared by recovering immune cells -- typically spleen cells or lymphocytes from lymph node tissue -- from immunized animals and immortalizing the cells in conventional fashion, *e.g.*, by fusion with myeloma cells or by Epstein-Barr (EB)-virus transformation and screening for clones expressing the desired antibody. The hybridoma technique described originally by Kohler and Milstein, Eur. J. Immunol., 6: 511 (1976) and also described by Hammerling *et al.*, In: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

It is possible to fuse cells of one species with another. However, it is preferable that the source of the immunized antibody-producing cells and the myeloma be from the same species.

The hybrid cell lines can be maintained in culture *in vitro* in cell culture media. The cell lines producing the antibodies can be selected and/or maintained in a composition comprising the continuous cell line in hypoxanthine-aminopterin thymidine (HAT) medium. In fact, once the hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cell lines can be stored and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody.

The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, ion-exchange chromatography, affinity chromatography, or the like. The antibodies described herein are also recovered from hybridoma cell cultures by conventional methods for purification of IgG or IgM, as the case may be, that heretofore have been used to purify these immunoglobulins from pooled plasma, *e.g.*, ethanol or polyethylene glycol precipitation procedures. The purified antibodies are sterile filtered.

While routinely mouse monoclonal antibodies are used, the invention is not so limited; in fact, human antibodies may be used and may prove to be preferable. Such antibodies can be obtained by using human hybridomas (Cote *et al.*, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 [1985]). In fact, according to the invention, techniques developed for the production of chimeric antibodies (Morrison *et al.*, Proc. Natl. Acad. Sci., 81: 6851 [1984]; Neuberger *et al.*, Nature, 312: 604 [1984]; Takeda *et al.*, Nature, 314: 452 [1985]; EP 184,187; EP 171,496; EP 173,494; PCT WO 86/01533; Shaw *et al.*, J. Nat. Canc. Inst., 80: 1553-1559 [1988]; Morrison, Science, 229: 1202-1207 [1985]; Oi *et al.*, BioTechniques, 4: 214 [1986]) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of

appropriate biological activity (such as ability to block activin's activity in hepatocytes) can be used; such antibodies are within the scope of this invention.

Techniques for creating recombinant DNA versions of the antigen-binding regions of antibody molecules (known as F(ab) fragments), which bypass the generation of monoclonal antibodies, are encompassed within the practice of this invention. One extracts antibody-specific messenger RNA molecules from immune system cells taken from an immunized animal, transcribes these into complementary DNA (cDNA), and clones the cDNA into a bacterial expression system. One example of such a technique suitable for the practice of this invention was developed by researchers at Scripps/Stratagene, and incorporates a proprietary bacteriophage lambda vector system that contains a leader sequence that causes the expressed F(ab) protein to migrate to the periplasmic space (between the bacterial cell membrane and the cell wall) or to be secreted. One can rapidly generate and screen great numbers of functional F(ab) fragments for those that bind the antigen. Such activin-binding molecules (F(ab) fragments with specificity for the activin polypeptide) are specifically encompassed within the term "antibody" as it is defined, discussed, and claimed herein.

One colorimetric test useful in determining if cell death has occurred is to measure reduction of MTT, as described by Carmichael *et al.*, Cancer Res., 47: 936-942 (1987). In this assay, if the cell is alive, its mitochondria will take up the dye MTT, resulting in a color change from yellow to purple. If the cell is dead, no color change will result.

The invention herein also encompasses "molecules with dual specificity for HGF and TGF-β or activin", which would include bispecific antibodies/immunoadhesins and bispecific linear molecules, such as the so-called "Janusin" structures recently reported by Traunecker *et al.*, EMBO, 10: 3655-3659 (1991). Such molecules with dual specificity for HGF and TGF-β or activin would comprise a domain having HGF binding activity and a domain having activin antagonist activity or TGF-β antagonist activity. In one embodiment the molecule is a single-chain polypeptide with an HGF binding activity in one domain and an activin antagonist amino acid sequence or a TGF-β antagonist amino acid sequence in the other domain. The activin-antagonist sequence may, for example be a follistatin sequence or a sequence comprising the antibody-antigen combining site of an anti-activin antibody. Similarly, the TGF-β-antagonist sequence may be originated from an anti-TGF-β antibody or a TGF-β-receptor.

If the two arms of the antibody-like immunoadhesin structure have two different specificities, the immunoadhesin is referred to as bispecific on the analogy of bispecific antibodies. In the present invention, one arm of the antibody-like, bispecific immunoadhesin structure is comprised of an HGF immunoglobulin chimera with the second arm comprised of an activin or TGF-β antagonist. Bispecific immunoadhesins can generally be assembled as hetero-multimers, and particularly as hetero-dimers, -trimers or -tetramers, essentially as disclosed in WO 89/02922 (published 6 April 1989), in EP 314,317 (published 3 May 1989), and in U.S. Patent No. 5,116,964 issued 2 May 1992.

Bispecific antibodies can, for example, be prepared by th s -called transfect ma method, essentially as described by Morris n, Science, 229: 1202-1207 (1985). This method is also suitable for the production of bispecific immunoadhesins, when a vector comprising the coding sequence of a chimeric (fusion) protein with a desired binding specificity is transfected into a hybridoma secreting an antibody providing the second specificity [see also Berg *et al.*, Proc. Natl. Acad. Sci. USA, 88: 4723 (1991)].

The recombinant production of bispecific immunoadhesins and antibodies is usually based on the co-expression of two immunoglobulin heavy chain-light chain pairs, where the two heavy chains have different specificities. Milstein and Cuello, Nature, 305: 537-539 (1983). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which the one having the correct bispecific structure needs to be isolated and purified.

According to an improved method disclosed in PCT Application No. PCT/US93/07783 filed 17 August 1993, trimeric bispecific immunoadhesins composed of a hybrid immunoglobulin heavy chain in one arm and a hybrid immunoglobulin heavy chain-light chain pair in the other arm are prepared. These immunoadhesins are preferably produced by individually introducing into suitable host cells the DNA sequences encoding the three immunoglobulin chains making up the trimeric molecule. As a result, the ratios of these DNA sequences can be freely changed. Notwithstanding the absence of the light chain in one arm and the asymmetric structure of the trimeric molecule, these molecules can be efficiently secreted in the form of correctly assembled and folded hetero-trimers. It was further found that the asymmetric structure facilitates the separation of the desired bispecific compound from unwanted immunoglobulin chain combinations, as the presence of an immunoglobulin light chain in only one half of the bispecific molecule provides for a facile way of separation.

For the purpose of the present invention, HGF can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the HGF product is combined in admixture with a pharmaceutically acceptable carrier. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo *et al.* These compositions will typically contain an effective amount of the HGF, for example, from on the order of about 0.5 to about 10 mg/ml, together with a suitable amount of carrier to prepare pharmaceutically acceptable compositions suitable for effective administration to the patient. HGF may be administered parenterally or by other methods that ensure its delivery to the bloodstream in an effective form.

Compositions particularly well suited for the clinical administration of HGF include sterile aqueous solutions or sterile hydratable powders such as lyophilized protein. Typically, an appropriate amount of a pharmaceutically acceptable salt is also used in the formulation to render the formulation isotonic.

Dosages and desired drug concentrations of such pharmaceutical compositions may vary depending on the particular use envisioned. A typical effective d s in rat experiments is about 250 μg/kg administered as an intravenous bolus injection. Interspecies scaling of dosages can be performed in a manner known in the art, e.g. as disclosed in Mordenti *et al.*, Pharmaceut. Res. 8: 1351 (1991) and in the references cited therein.

Typically, the activin or TGF-β antagonist used in the method of this invention is formulated by mixing it at ambient temperature at the appropriate pH, and at the desired degree of urity, with pharmaceutically acceptable carriers, *i.e.*, carriers that are non-toxic to recipients at the dosages and concentrations employed. Suitable carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Co., edited by Oslo *et al.* These compositions will typically contain an effective amount of the activin antagonist, for example, from on the order of about 0.5 to about 10 mg/ml, together with a suitable amount of carrier to prepare pharmaceutically acceptable compositions suitable for effective administration to the patient.

The pH of the formulation depends mainly on the particular type and the concentration of antagonist, but preferably ranges anywhere from about 3 to about 8. Formulation in an acetate buffer at pH 5 is a suitable embodiment.

Compositions particularly well suited for the clinical administration of activin antagonist include sterile aqueous solutions or sterile hydratable powders such as lyophilized protein. Typically, an appropriate amount of a pharmaceutically acceptable salt is also used in the formulation to render the formulation isotonic.

Sterility is readily accomplished by sterile filtration through (0.2 micron) membranes. Activin antagonist ordinarily will be stored as an aqueous solution, although lyophilized formulations for reconstitution are acceptable.

The antagonist composition will be formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The "therapeutically effective amount" of activin antagonist to be administered will be governed by such considerations, and is the minimum amount necessary to prevent, ameliorate, or treat the protein-mediated liver disorder. Such amount is preferably below the amount that is toxic to the mammal or renders the mammal significantly more susceptible to infections.

As a general proposition, the pharmaceutically effective amount of the activin or TGF-β antagonist administered parenterally per dose will be in the range of about 0.01 to 100 mg/kg of patient body weight per day, with the typical range of activin antagonist used being about 0.1 to 50 mg/kg/day. Interspecies scaling of dosages can be performed in a manner known

in the art, *e.g.*, as disclosed in Mordenti *et al.*, Pharmaceut. Res., 8: 1351 (1991) and in the references cited therein.

HGF and an activin or TGF-β antagonist may be formulated together in a single composition comprising therapeutically effective amounts of each of HGF and antagonist in a pharmaceutically acceptable carrier having appropriate pH for effective administration to the patient. Respective formulations of HGF and the activin or TGF-β antagonist may be combined in vitro before administration or separately administered simultaneously or in tandem, in either order, with any second administration taking place preferably within about 1-24 hours of the first administration and more preferably within about 1-5 hours of the first administration.

The compounds are usually administered as pharmaceutical compositions, usually formulated in dosage forms by methods known in the art; for example, see Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania, 15th Edition 1975. For parenteral administration, HGF and the activin or TGF-β antagonist are typically formulated in the form of injectable solutions, suspensions or emulsions, in admixture with a suitable pharmaceutically acceptable vehicle and optionally other pharmaceutically acceptable additives. Typical vehicles include saline, dextrose solution, Ringer's solution, etc., but non-aqueous vehicles may also be used.

The term "antidote" as used herein refers to those substances which antagonize the effects of hepatotoxic compounds by inhibiting the binding of a hepatotoxic compound to its receptor, causing a physiological response that opposes the actions of a hepatotoxic compound, changing the chemical nature of a poison to a less toxic form, or decreasing the amount of hepatotoxic compound that reaches its site of action by either preventing its absorption or enhancing its elimination or metabolism. Antidotes are available for only a limited number of hepatotoxic compounds (Smith, C. Textbook of Pharmacology, pg. 998 (1992)).

The use of the term "hepatotoxic compound" herein refers to any compound, drug, chemical, or element capable of inducing liver damage upon exposure to the liver.

The term "administration" or "administered" as used herein in reference to HGF refers to that administration of HGF which occurs prior to, simultaneous with, or after administration of or exposure to a hepatotoxic compound, clinical therapy-inducing liver damage, radiation, or other means inducing liver damage. HGF may be combined in vitro with a hepatotoxic compound before administration or separately administered simultaneously or in tandem, in either order, with any second administration taking place generally within about 6 hours of the first administration.

HGF or an activin antagonist may be administered to a subject mammal, preferably human, via any of the accepted modes of administration for agents which exhibit such activity. These methods include subcutaneous and, preferably, parenteral administration.

Examples of parenteral administration routes are intravenous, intrapulmonary, intraarterial, intramuscular, and intraperitoneal administration, the intravenous route being preferred. Administration may be continuous or bolus dosing in sufficient amounts to maintain therapeutically effective levels.

HGF may be administered to a subject mammal alone according to the present invention, or combined with other therapies effective in the prevention or treatment of liver damage, such as vascular endothelial growth factor (VEGF) or other growth factors, proteins with growth factor-like activities, such as cytokines or cytokine antagonists or tissue plasminogen activator or other therapeutics.

The use of the term "growth factor" as used herein refers to those factors required to regulate developmental events or required to regulate expression of genes encoding other secreted proteins that may participate in intercellular communication and coordination of development and includes, but is not limited to, insulin-like growth factor-I and II (IGF-I and II), epidermal growth factor (EGF), type a and type b transforming growth factor (TGF-α and TGF-β), epidermal growth factor (EGF), nerve growth factor (NGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), sarcoma growth factor (SGF), granulocyte-macrophage colony stimulating growth factor (GM-CSF), vascular endothelial growth factor (VEGF), and hemopoietic growth factors.

VEGF is a specific mitogen for endothelial cells that acts to increase microvascular permeability. VEGF is expressed in many normal adult organs, including, lung, kidney, adrenal gland, heart, liver, and stomach mucosa, as well as in elicited peritoneal macrophages. Berse *et al.*, Supra demonstrate particularly high VEGF mRNA levels in several human tumors, where it may be involved in promoting tumor angiogenesis and stroma generation, both as an endothelial cell mitogen and indirectly by its permeability enhancing effect that leads to the deposition of a provisional fibrin gel matrix. The mRNA sequence of VEGF is described in Leung *et al.*, (Science 246: 1306-1309 (1989)).

Tissue plasminogen activator (tPA) is an enzyme that has the potent ability to dissolve blood clots *in vivo* and is used as a therapeutic in the treatment of vascular diseases, such as myocardial infarction. A substantially pure form of tPA was first produced from a natural source and tested for *in vivo* activity by Collen *et al.*, U.S. patent number 4,752,603. Pennica *et al.*, (Nature 301: 214 (1983)) determined the DNA sequence of tPA and deduced the amino acid sequence from this DNA sequence (see U.S. patent number 4,766,075, issued 23 August 1988). TPA has been shown to be useful in the treatment of veno occlusive disease (VOD) (Baglin *et al.*, Bone Marrow Transplant 5(6): 439-441 (1990)) and (Rosti *et al.*, Lancet 339: 1481-1482 (1992)).

Cytokines are secreted peptides or proteins that regulate the intermediary metabolism of amino acids, proteins, carbohydrates, lipids and minerals. Cytokines include peptides or proteins that act to mediate inflammation and are involved in intercellular communication

modulating cell proliferation, and adhesion f inflammatory cells t the walls of the vessels, and to the extra cellular matrix. Cytokines are essential for the communication between the liver and extrahepatic sites and within the liver itself. Cytokines interact with hormones such as glucocordicoids, resulting in a complex network of mutual control. Many cytokines exert growth factor-like activity in addition to their specific proinflammatory effects. The liver is an important site of cytokine synthesis and the major clearance organ for several cytokines. In liver disease, cytokines are involved in the onset of intrahepatic immune responses, in liver regeneration, and in the fibrotic and cirrhotic transformation of the liver (Andus *et al.*, Hepatology 13(2): 364-375 (1991)). Cytokines include, but are not limited to, the interleukin family of peptides and proteins; interferons-alpha,-beta,gamma; tumor necrosis factors-alpha and -beta; and prostaglandins E1 and E2.

The use of the term "therapeutic" as used herein refers to those agents effective in the prevention or treatment of a disorder or pathologic physiological condition.

Further details of the invention are illustrated in the following non-limiting example.

EXAMPLE 1

A. Recombinant Production of hHGF

Recombinant hHGF (rhHGF) was produced as described in PCT Publication No. WO92/22321.

An hHGF cDNA clone (HLC3) isolated from a human leukocyte library as described by Seki *et al.*, Supra, was cloned into the broadly applicable parental expression vector pSVI6B5. pSVI6B5 carries polylinker regions which provide convenient, unique restriction endonuclease recognition sites that can be used to introduce any sequence that encodes a polypeptide of interest.

CHO-dhfr$^-$ cells (Urlaub *et al.*, Proc. Natl. cad. Sci. USA 77: 4216-4220 (1980)) were contransfected with the above-described pSVI6B5-based hHGF expression vector and with a dhfr selection vector pFD11 (Simonsen and Levinson, Proc. Natl. Acad. Sci. USA 80: 2495-2499 (1983)) using the general procedure of Graham and van des Eb, Virology 52: 456-467 (1973)). The latter plasmid encodes DHFR, thereby conferring methotrexate resistance on the transfected cells and allowing for selection of hHGF expressing transformants. The transformed dhfr$^-$ cells were selected by growth in glycine-, hypoxanthine- and thymidine-deficient medium. Colonies that arose on this selection medium were isolated using cotton swabs and propagated in the same medium to several generations. After cell growth, the cells were amplified and selected with increasing amounts of methotrexate using standard techniques. Clones that could grow in selective media, and therefore incorporated the transfected DHFR containing plasmid, were screened for the presence of secreted HGF. HGF activity in the media of these clones was assayed with the mitogenic assay described hereinbelow. Alternatively, HGF activity in culture media may also be measured by incorporation of ^{125}I-labelled deoxyuridine into rat hepatocyte in primary

culture as described by Nakamura *et al.*, Nature 342, 440-443 (1989). hHGF was purified essentially as described by Nakamura *et al.*, Supra.

B. Protection from Hepatotoxicity by Treatment with rhHGF

We have examined the effects of HGF in combination therapy with BiCNU®-Carmustine (Bristol-Myers Squibb Company, Oncology division), in male F344 rats, body weighing 190-260 grams each.

Carmustine chemically is 1,3-bis (2-chloroethyl)-1-nitrosourea and belongs to a group of chemotherapeutics used in the treatment of certain neoplastic diseases. BiCNU® is used in brain tumors, both primary and metastatic; multiple myeloma; Hodgkin's disease, as a secondary therapy; and non-Hodgkin's lymphoma, as a secondary therapy.

One adverse reaction to BiCNU® is hepatotoxicity manifested by increased transaminase, alkaline phosphatase and bilirubin levels Patients receiving high dose treatment (usually with bone marrow transplantation) are in danger of developing hepatic veno-occlusive disease (VOD) which will present with hepatomegaly (enlargement of the liver) and ascites (accumulation of fluid). These findings are clinically similar to Budd-Chiari syndrome. About 20% of bone marrow transplantation patients develop this syndrome and in about 47% of these patients the severe form of VOD is fatal. Other adverse reactions include delayed cumulative myelosuppression, thrombocytopenia more severe than leucopenia and anemia, dose dependent pulmonary toxicity characterized by pulmonary fibrosis with delayed onset (even years), and nephrotoxicity, with progressive azotemia and decrease in kidney size and renal failure.

BiCNU® is supplied as lyophilized yellow flakes with a molecular weight of 214.06. It is soluble in lipids and alcohol. For human use, after reconstitution of 100mg of BiCNU® in 3 mls of ethanol, 27 mls of sterile water is added for injection purposes and the drug is administered intravenously.

Protocol:

The concentration of rhHGF used was 2.45 mg/ml, and the dose was 280 ug/kg of body weight delivered in 0.25 ml of Vehicle (phosphate buffered saline (PBS) +0.1% bovine serum albumin (BSA), sterilized) injected intravenously (IV) at -30 min., 6, 12, 24, 30, and 36 hours.

In the rat, the dose of BiCNU®- was 50 mg/kg of body weight administered in a single intraperitoneal (IP) injection at 0 hours. The vehicle was peanut oil and the whole dose was delivered in 1.5 ml. Using the toxicokinetic scaling method of Mordenti et al.,# ("The Use of Interspecies Scaling in Toxicokinetics", Toxicokinetics and New Drug Development, A. Yacobi *et al.*, eds. Pergamon Press, New York p42-96 [1989]) and Chappel et al.("Extrapolation of Toxicological and Pharmacological Data from Animals to Humans", Advances in Drug Research, Vol 20 B.Testa, Ed., Academic Press, San Diego, pp1-116 [1991]) the 50 mg/kg

dose in rats equals a 9.2 mg/kg dose in humans. Clinical dose of BiCNU® used in humans is 5-15 mg/kg.

Samples were collected at 48 hours after IP injection of BiCNU®. One set of 7 rats received a combination of rhHGF with BiCNU , and one set of 7 rats received BiCNU® plus the rhHGF vehicle. One set of 7 animals received I.P. peanut oil plus the rhHGF vehicle and served as controls.

Results:

Animals receiving combination therapy of BiCNU® and rhHGF showed decreased levels of total bilirubin, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, and g-glutamyl transpeptidase from those animals receiving BiCNU® alone as shown in Figure 1.

Protocol for the liver histopathology study:

One set of 7 animals received I.P. peanut oil only and served as controls. Fourteen other rats were inoculated with BiCNU® in peanut oil at 50 mg/kg body weight, and 7 of the 14 rats received rhHGF (280 μg /kg body weight I.V. at -30 minutes, 6, 12, 24, 30, and 36 hours. All rats were euthanasia by CO_2 at 48 hours after inoculation of BiCNU® or oil I.P. Sections of liver, sternum, lung, kidney, and spleen were fixed in formalin, sectioned in paraffin, stained wih H & E, and examined histologically.

Histopathology Results:

Lesions were consistently present in the liver, bone marrow, and spleen of rats which received BiCNU®. BiCNU® induces biliary necrosis and hepatocellular necrosis in rats. Rats which receive rhHGF have biliary necrosis comparable to that in untreated rats and rhHGF reduces the severity of hepatocellular necrosis seen in rats treated with BiCNU® alone, as shown in Figure 2.

EXAMPLE 2

This example shows HGF protection against Activin and TGF-b induced Hepatocyte death.

Method

Hepatocytes were obtained from adult female Sprague-Dawley rats by collagenase perfusion, as described by Garrison and Haynes. The cells were plated at a density of 4000 cell/well in 96-well microtiter plates (Falcon). The culture medium was William's E medium supplemented with penicillin (100 U/ml), streptomycin sulfate (100 μg/ml), L0glutamine (2mM), transferrin (10 μg/ml), and trace elements (0.01%). The cells were plated in medium containing 5% fetal bovine serum at 37°C in 5% CO_2. After 16 hours, the plating medium was replaced with 100 μl serum-free medium containing: no additions for the control; HGF at 10, 100, 1000 ng/ml; activin-A (10 ng/ml) alone or in combination with HGF at 10, 100, 1000, ng/ml; or TGF-b (1 ng/ml) alone or in combination with HGF at 10, 100, 1000 ng/ml.

Twenty four hours later, viability was assessed by measuring the reduction of MTT, an index of mitochondrial function, essentially as described by Carmichael et al. (Cancer Res 47:936-942 [1987]). MTT was dissolved to 5 mg/ml in phosphate-buffered saline and 5 µl was immediately added to each well. After incubation at 37°C for 4 hours, the media was removed by gently inverting the plate and blotting on a paper towel. The cells were solubilized by addition of 100 µl DMSO followed by shaking for 5 minutes on an orbital shaker. The absorbance at 560 nm, less the absorbance at the reference wavelength of 690 nm, was measured in an automatic plate reader (SLT Lab Instruments). In some experiments in which cells were cultured in more than one microtiter plate, data were normalized to the controls in each plate.

Results

As shown in the Figure 3, HGF causes a small increase in viability in control cultures, as measured by MTT reduction, which is an index of mitochondrial function. Activin caused viability to be reduced by about 70%, but this effect was substantially abrogated if the culture medium also contained HGF. Similarly, TGF-b caused a large decrease in hepatocyte viability, and as with activin, this effect was largely prevented by inclusion of HGF.

EXAMPLE 3

This example shows the use of HGF co-administered with an activin antagonist to provide protection from liver damage.

Methods

A transgenic mouse expressing hepatitis B virus proteins is used to determine the preventative effect provided by co-administration of HGF with follistatin. Suitable transgenic mice are the two generically different categories of HBV-transgenic mice (lineages 23-3 and 80-219) described and used in Gilles *et al.*, (J Virol., 66:3955-3960 (1992)).

Recombinant HGF is produced as described in Example 1. The concentration of rhHGF used is 2.45 mg/ml and the dose is 280 µg/kg of body weight delivered in 0.25 ml of Vehicle (phosphate buffered saline (PBS) + 0.1% bovine serum albumin (BSA, sterilized) and injected intravenously (IV). The dose of follistatin used is in the range of about 0.1 to 100 mg / kg of patient body weight per day and is delivered by intravenous injection (IV). Interspecies scaling of dosages can be performed in a manner known in the art, e.g., as disclosed in Mordenti et al Supra and in the references cited therein.

One set of animals receives vehicle only and serves as a control. Another set of animals is injected with rhHGF every 6 hours over a 10 day period. A third set of animals is injected with rhHGF and follistatin in combination every 6 hours over a 5 day period. After 1, 3, and 5, days of injections, the mice were anesthetized with ketamine-xylazine, and blood was collected by cardiac puncture and allowed to clot for one hour at room temperature. Serum aliquots are stored at -70°C prior to measurement of bilirubin, ALT, and AST on a Monarch Model 7000 automated analyzer. The liver is dissected free from connective tissue

and weighed, and pieces are fixed in neutral buffer f rmalin. Paraffin-emb dded sections are cut at 4 μm stained with hematoxylin and eosin and examined histologically.

It is reasonably expected that the transgenic mouse data resulting from Example 3 may be extrapolated to horses, cows, and other mammals, correcting for the body weight of the mammal in accordance with recognized veterinary and clinical procedures. Using standard protocols and procedures, the veterinarian or clinician will be able to adjust the doses, scheduling, and mode of administration of the HGF and activin antagonist to achieve maximal effects in the desired mammal being treated. Humans are believed to respond in this manner as well.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those ordinarily skilled in the art that various modifications may be made to the disclosed embodiments without diverting from the overall concept of the invention. All such modifications are intended to be within the scope of the present invention.

CLAIMS:

1. A method for the prevention of the establishment or progress of liver damage in a patient at risk of developing or having been diagnosed with liver damage comprising administering to said patient a preventatively effective amount of hepatocyte growth factor (HGF).

2. The method of claim 1 wherein said patient is mammalian.

3. The method of claim 2 wherein said patient is human and human HGF (hHGF) is administered.

4. The method of claim 3 wherein hHGF is administered prior to the diagnosis of liver damage.

5. The method of claim 3 wherein hHGF is administered before liver damage occurs.

6. The method of claim 3 wherein said patient is at risk of developing or has developed liver damage due to exposure to a hepatotoxic compound.

7. The method of claim 6 wherein hHGF administration is initiated prior to the exposure to said hepatotoxic compound.

8. The method of claim 7 wherein hHGF is administered concurrently with the administration of said hepatotoxic compound.

9. The method of claim 7 or claim 8 wherein hHGF administration is maintained after the administration of said hepatotoxic compound is terminated.

10. The method of claim 6 wherein the patient has been diagnosed with veno occlusive disease.

11. A composition comprising a therapeutically effective amount of a hepatotoxic compound and a liver damage preventative amount of HGF.

12. The composition of claim 11 wherein said HGF is hHGF.

13. The composition of claim 12 wherein said hepatotoxic compound and said hHGF are in separate pharmaceutical formulations each comprising at least one pharmaceutically acceptable excipient.

14. A method for the treatment of a patient with a hepatotoxic therapeutic agent effective in the prevention or treatment of a disorder or pathologic physiological condition, comprising:

a) administering to said patient, simultaneously or in optional order, a biologically effective dose of said therapeutic agent and a preventatively effective amount of HGF,

b) monitoring said patient for indication of liver damage, and

c) continuing said treatment until said disorder or condition is eliminated or until liver damage is indicated.

15. A method for the prevention of the establishment or progress of liver damage in a patient at risk for developing or having been diagnosed with viral or autoimmune hepatitis

comprising administering to said patient a liver damage preventative amount of hepatocyte growth factor (HGF).

16. The method of claim 15 further comprising administering to said patient a therapeutically effective amount of an activin antagonist.

17. The method of claim 16 wherein said activin antagonist is follistatin.

18. The method of claim 16 wherein said activin antagonist is an anti-activin antibody.

19. The method of claim 16 wherein said activin antagonist is a soluble form of an activin receptor.

20. The method claim 15 further comprising administering to said patient a therapeutically effective amount of a TGF-β antagonist. 21. The method of claim 20 wherein said TGF-β antagonist is an anti-TGF-β antibody.

22. The method of claim 20 wherein said TGF-β antagonist is a soluble form of the TGF-β receptor.

23. A composition comprising a liver damage preventative amount of HGF and a therapeutically effective amount of an activin antagonist.

24. The composition of claim 23 wherein said HGF is hHGF.

25. The composition of claim 23 wherein the therapeutically effective amount of said activin antagonist and said preventative amount of HGF are in separate pharmaceutical formulations each comprising at least one pharmaceutically acceptable excipient.

26. A composition comprising a liver damage preventative amount of HGF and a therapeutically effective amount of an TGF-β antagonist.

27. The composition of claim 26 wherein the therapeutically effective amount of said TGF-β antagonist and said preventative amount of HGF are in separate pharmaceutical formulations each comprising at least one pharmaceutically acceptable excipient.

28. A molecule with dual specificity for HGF and activin or TGF-β comprising a first domain having HGF biological activity and a second domain having activin antagonist biological activity or TGF-β antagonist biological activity.

29. The molecule of claim 28 that is a single-chain polypeptide with an HGF amino acid sequence in the first domain and an activin antagonist activity or TGF-β antagonist activity in the second domain.

30. The molecule of claim 29 that comprising an immunoglobulin sequence.

31. The molecule of claim 30 wherein the second domain is an anti-activin antibody or an anti-TGF-β antibody.

32. The molecule of claim 28 that is a bispecific immunoadhesin comprising an HGF amino acid sequence and a TGF-β-antagonist sequence or an activin-antagonist sequence fused to an immunoglobulin sequence.

33. The use of HGF in the preparation of a pharmaceutical composition for the prevention of liver damage comprising admixing HGF with a pharmaceutically acceptable excipient.

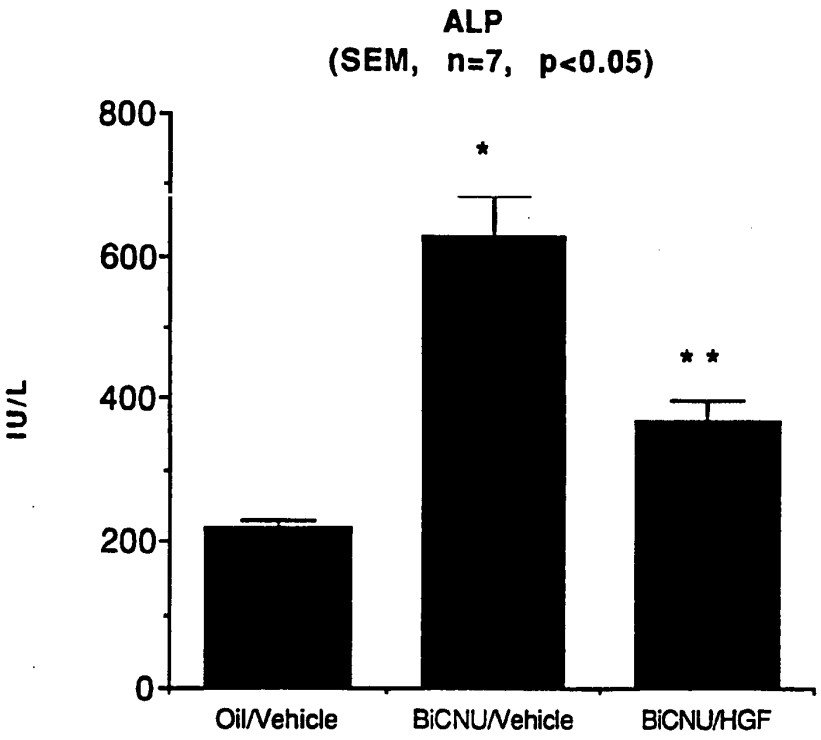
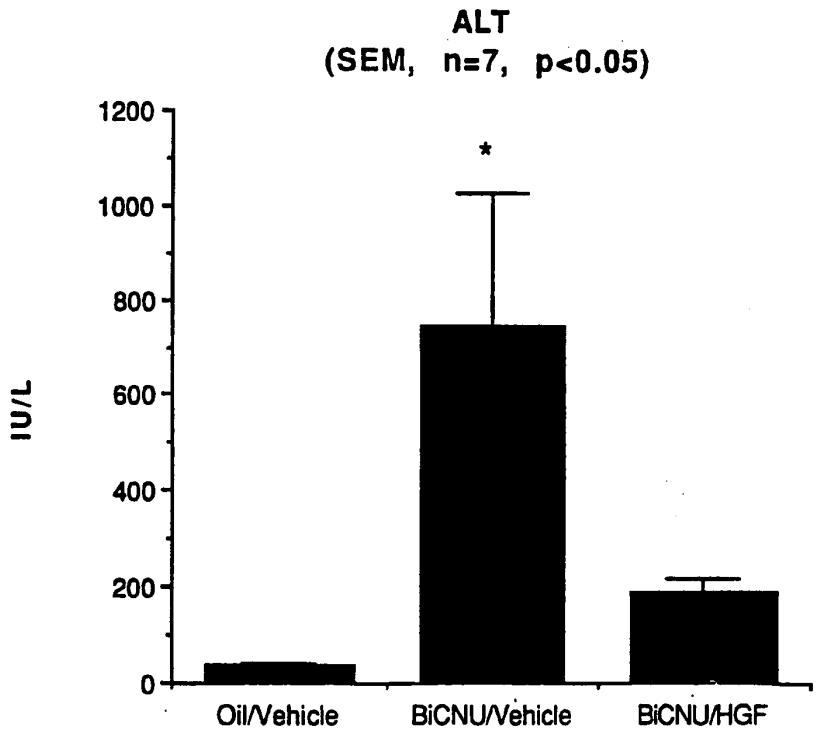

ALP
(SEM, n=7, p<0.05)
800
600
400
200
0
IU/L
*
* *
Oil/Vehicle
BiCNU/Vehicle
BiCNU/HGF

FIG.1A


ALT
(SEM, n=7, p<0.05)
1200
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800
600
400
200
0
IU/L
*
Oil/Vehicle
BiCNU/Vehicle
BiCNU/HGF

FIG.1B

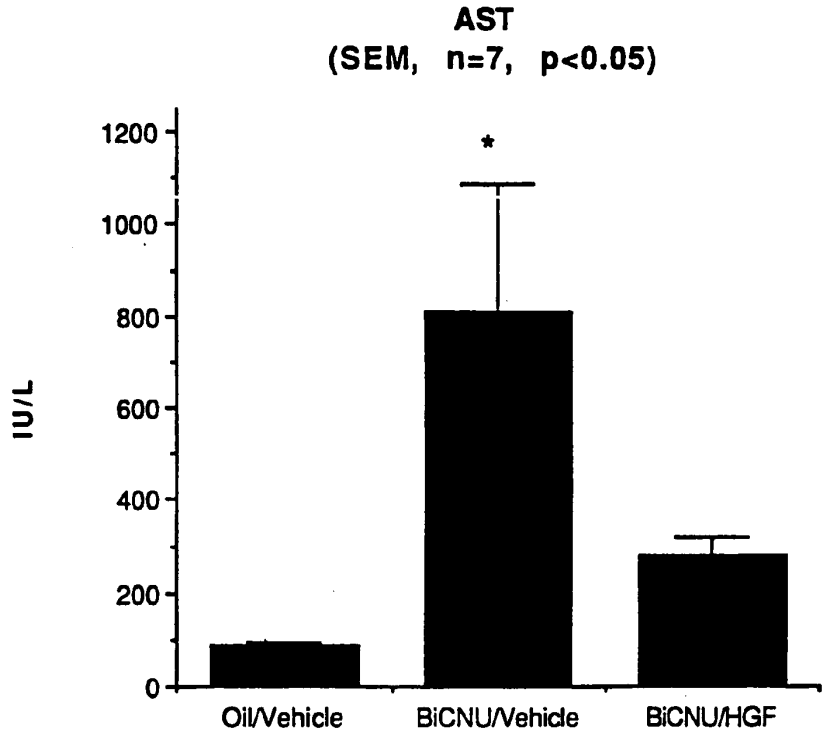
AST
(SEM, n=7, p<0.05)
IU/L
1200
1000
800
600
400
200
0
*
Oil/Vehicle
BiCNU/Vehicle
BiCNU/HGF

FIG. 1C

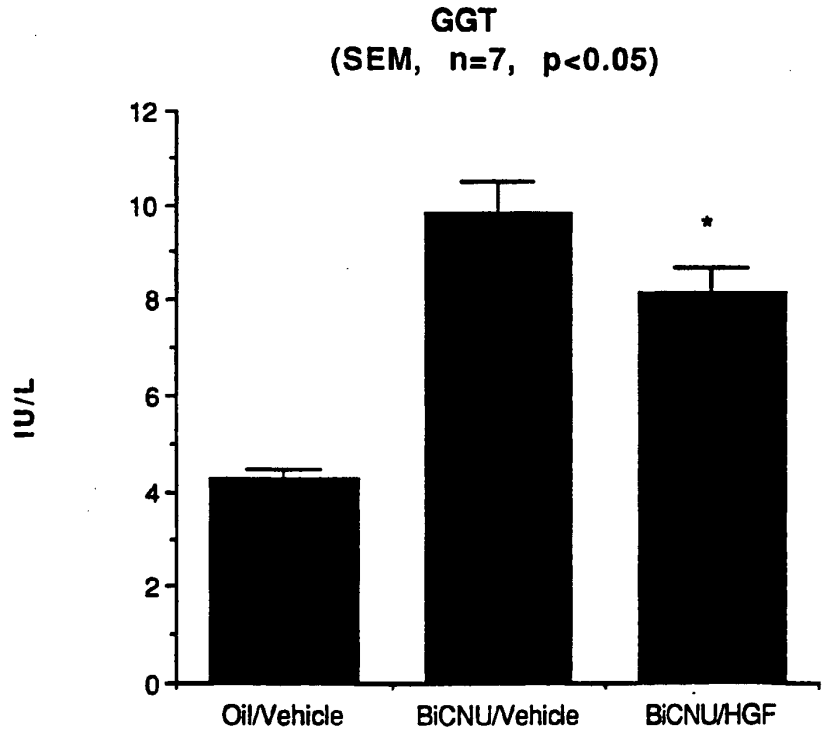
GGT
(SEM, n=7, p<0.05)
IU/L
12
10
8
6
4
2
0
*
Oil/Vehicle
BiCNU/Vehicle
BiCNU/HGF

FIG. 1D

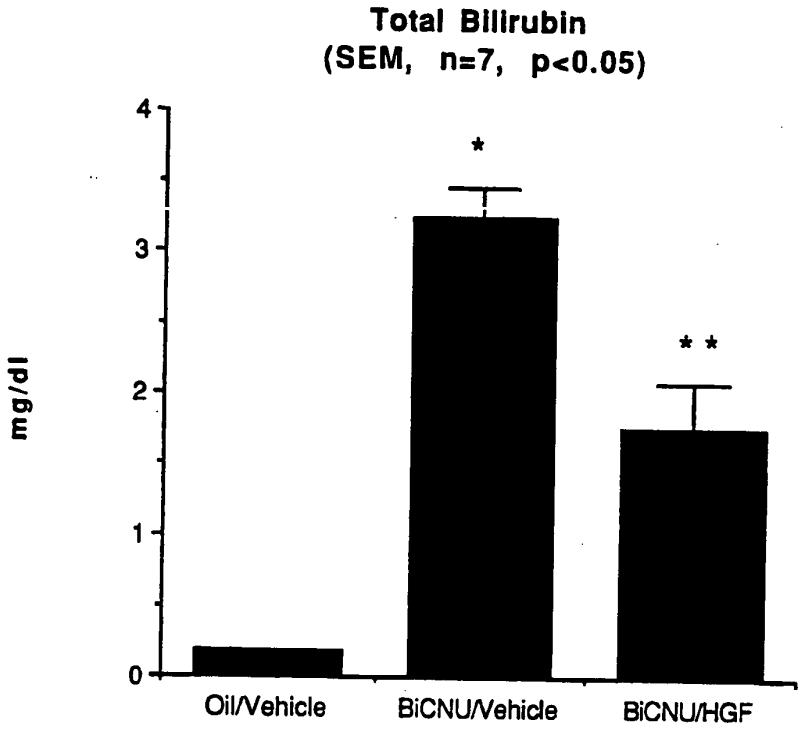

FIG. 1E

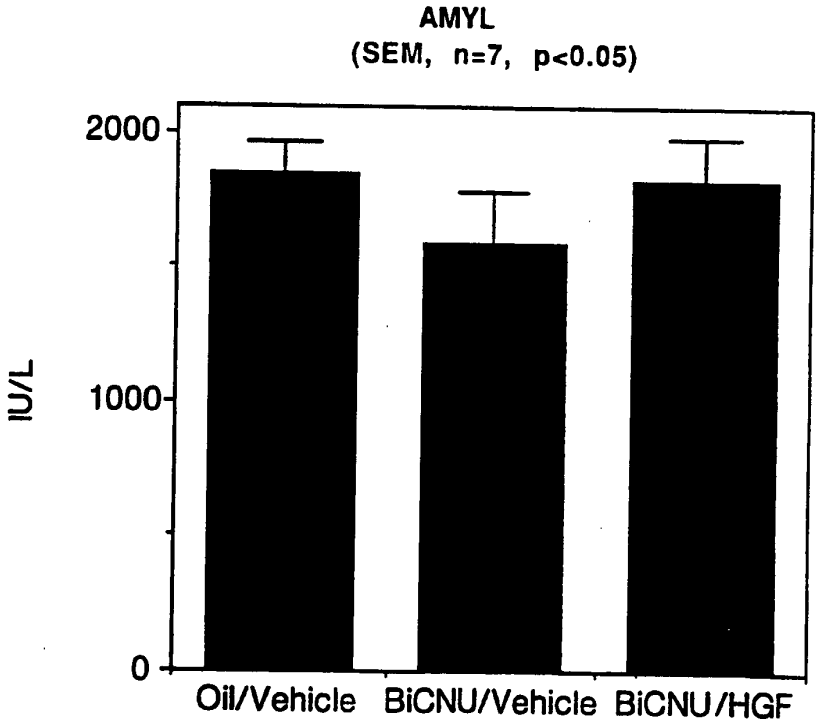

FIG. 1F

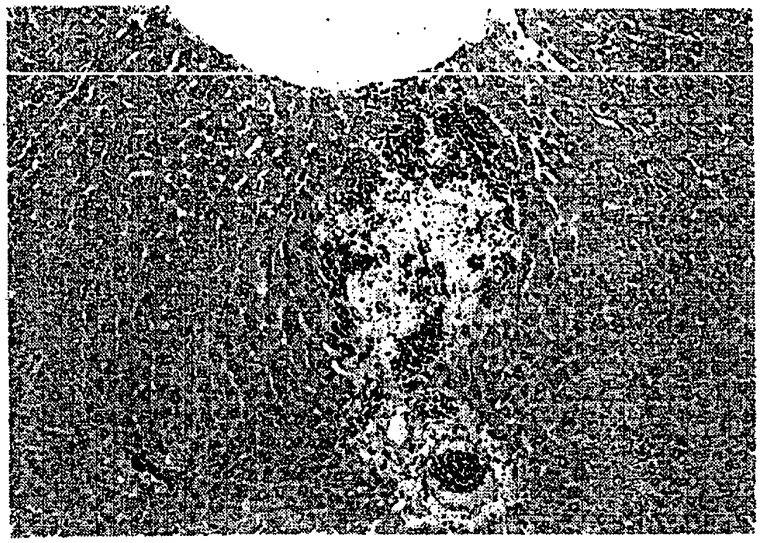

FIG.2A

FIG.2B

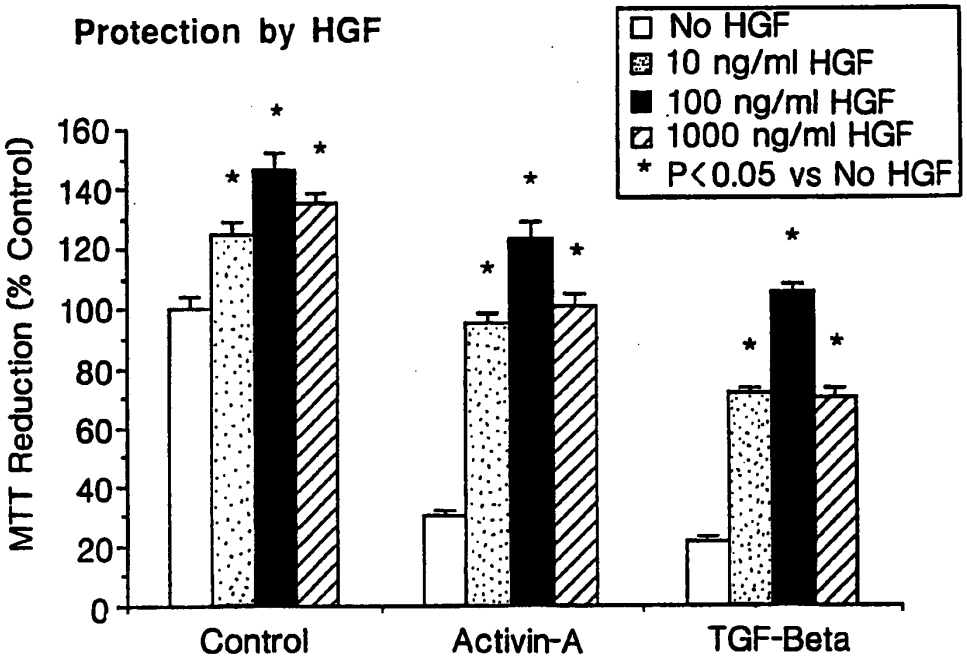
Protection by HGF
No HGF
10 ng/ml HGF
100 ng/ml HGF
1000 ng/ml HGF
* P<0.05 vs No HGF
MTT Reduction (% Control)
160
140
120
100
80
60
40
20
0
Control
Activin-A
TGF-Beta

FIG.3

INTERNATIONAL SEARCH REPORT

Internati Application No
PCT/US 93/08718

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 A61K37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO,A,93 08821 (NAKAMURA, TOSHIKAZU ET AL.) 13 May 1993 ABSTRACT ---	1-4,33
X	DATABASE WPI Week 9103, Derwent Publications Ltd., London, GB; AN 91-017990 & JP,A,2 288 899 (TOYOBO KK) 28 November 1990 see abstract --- -/--	1-4,33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 1993	24 -01- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Rempp, G

Form PCT/ISA/210 (second sheet) (July 1992)

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Week 9142, Derwent Publications Ltd., London, GB; AN 91-206734 & JP,A,3 204 899 (OTSUKA PHARM KK) 6 September 1991 see abstract	1-4,33
X	DATABASE WPI Week 9211, Derwent Publications Ltd., London, GB; AN 92-085905 & JP,A,4 030 000 (TOYOBO KK) 31 January 1992 see abstract	1-4,33
X	EP,A,0 456 188 (NAKAMURA, TOSHIKAZU ET AL.) 13 November 1991 see page 9, line 32 - page 10, line 36	1-4,33
X,P	WO,A,92 22321 (GENENTECH, INC.) 23 December 1992 cited in the application	1-16

INTERNATIONAL SEARCH REPORT

I national application No.
PCT/US 93/ 08718

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 1-10, 14-22 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REP RT

Information on patent family members

Internatio \pplication No
PCT/US 93/08718

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9308821	13-05-93	NONE		
EP-A-0456188	13-11-91	JP-A-	4018028	22-01-92
WO-A-9222321	23-12-92	US-A-	5227158	13-07-93
		AU-A-	2144192	12-01-93